Copper complexes of (−)-epicatechin gallate and (−)-epigallocatechin gallate act as inhibitors of Ribonuclease A

Kalyan Sundar Ghosh, Tushar Kanti Maiti, Abhishek Mandal, Swagata Dasgupta*

Department of Chemistry, Indian Institute of Technology, Kharagpur 721 302, India

Received 28 June 2006; accepted 12 July 2006

Available online 25 July 2006

Edited by Miguel De la Rosa

Abstract Green tea polyphenols, which have the ability to inhibit angiogenesis, form complexes with Cu(II), a known potent stimulator of blood vessel proliferation. Copper complexes of (−)-epicatechin gallate and (−)-epigallocatechin gallate were found to inhibit the enzymatic activity of Ribonuclease A (RNase A) as revealed by an agarose gel based assay and urea denatured gel electrophoresis. The copper complexes were found to be non-competitive inhibitors of RNase A with inhibition constants in the micromolar range. Changes in the secondary structure of the protein are found to occur due to the interaction as revealed from Fourier transform infrared and circular dichroism studies.

1. Introduction

Cellular RNA levels are controlled post-transcriptionally by ribonucleases of varying specificity [1]. Angiogenin [2], eosinophil-derived neurotoxin (EDN) [3] and bovine seminal Ribonuclease [4] are among the myriad of proteins with ribonuclease activity having significant homology to RNase A in addition to their biological activities [5]. Angiogenin, a member of the ribonuclease superfamily showing ~60% homology with RNase A [6], is a potent inducer of angiogenesis, the process of new blood vessel growth. We have investigated the effect of green tea polyphenols isolated from green tea leaves on angiogenin induced angiogenesis [7]. Subsequent studies indicated that the major green tea polyphenol, (−)-epigallocatechin gallate inhibited the ribonucleolytic activity of RNase A in a non-competitive manner [8].

The anticancer and antiangiogenic activities of the major green tea polyphenols; (−)-epicatechin (EC), (−)-epicatechin gallate (EGC), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG) [9] (Fig. 1) are well known [10,11]. Copper, on the other hand, an essential trace element present in the diet, is an important cofactor for angiogenesis [12]. A number of drugs have been used to reduce copper levels in blood via chelation. Penicillamine [13], captopril [14] and ammonium tetrathiomolybdate [15] are known chelators of copper that have been used for antiangiogenic therapy, though side effects are also observed [16]. Thus copper complexation by naturally occurring compounds from dietary constituents that have the ability to inhibit angiogenesis on their own can be effectively exploited in the development of novel antiangiogenic compounds.

We have investigated the effect of the copper complexes of ECG and EGCG on the enzymatic activity of RNase A, since the presence of the gallate moiety has shown to have an inhibitory effect on several proteins [17–20]. Agarose gel and urea denatured gel electrophoresis experiments were conducted followed by kinetic experiments. Secondary structural changes occurring due to the interaction of the complexes with RNase A were studied by Fourier transform infrared and circular dichroism studies.

2. Materials and methods

2.1. Materials

RNase A, yeast transfer ribonucleic acid (tRNA), cytidine 2′,3′ cyclic monophosphate (2′,3′-cCMP), ECG and EGCG were from Sigma–Aldrich, D2O from Acros Organics and other reagents from SRL India. A Perkin–Elmer UV–Vis spectrophotometer (Lambda 25) was used for UV measurements. Concentrations were determined using the following data: RNase A ε278.5 = 9800 M⁻¹ cm⁻¹ [21]; 2′,3′-cCMP ε268 = 8500 M⁻¹ cm⁻¹ [22]; ECG and EGCG, ε278 = 12443 and 11920 M⁻¹ cm⁻¹ [23], respectively.

2.2. Complexation of ECG and EGCG with Cu(II) by UV spectroscopy

The absorption spectra were obtained on successive addition of 0.58 mM CuSO4 to 0.057 mM ECG and 0.17 mM EGCG in 20 mM acetate buffer, pH 5.0, to reach metal/polyphenol ratios ranging from 0.2:1 to 4.4:1 and 0.2:1 to 6.8:1, respectively. The stoichiometry of the polyphenol–metal complex was determined by the mole-ratio method using a Jobs plot. The absorbance of each solution was measured at 270 nm (λmax of the polyphenols) and plotted against the mole fraction of the respective polyphenol. The breakpoint in the plot corresponds to the mole fraction of the polyphenol in its metal complex giving the binding stoichiometry.

2.3. Agarose gel-based assay

Inhibition of RNase A by the copper complexes was checked qualitatively by the degradation of tRNA in an agarose gel. 20 μl of RNase A (3.43 μM) was mixed with 20 μl each of 0.027 mM ECG, EGCG and their copper complexes and the resulting solutions were incubated for 6 h. 20 μl aliquots of the incubated mixtures were then mixed with

0001-5793/38.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2006.07.054
20 μl of tRNA solution (7.5 mg/ml tRNA, freshly dissolved in RNase free water) and incubated for another 30 min. 10 μl of sample buffer which contains 10% glycerol and 0.025% bromophenol blue was added to the mixture. 15 μl from each solution was extracted and loaded onto a 1.1% agarose gel. The undegraded tRNA was visualized by ethidium bromide staining under UV light.

2.4. Urea denatured gel electrophoresis

20 μl of 0.97 μM of RNase A was incubated with 20 μl of 0.1 mM ECG, EGCG and their copper complexes for 3 h. 20 μl of each incubated mixture was taken and mixed with 20 μl tRNA (5.0 mg/ml) solution and incubated for another 30 min. 15 μl of the gel loading buffer (0.01% bromophenol blue and 10% glycerol in 1× TAE buffer, pH 8.0) was added to each incubated solutions. 15 μl from each mixture was loaded onto a 15% polyacrylamide gel consisting of 7 M urea. The gel was visualized after fixing with 7.5% acetic acid and silver staining.

2.5. Precipitation assay

The inhibition of the ribonucleolytic activity of RNase A was assayed by the precipitation method as described by Bond [24]. 10 μl of RNase A (5.6 μM) was mixed with 0–0.061 mM of the complexes and incubated for 2 h. 20 μl of the solutions were then mixed with 40 μl of tRNA, 40 μl of phosphate buffer of pH 6.5 containing 5 mM EDTA and 0.5 mg/ml HSA. After incubation at 25 °C for 30 min, 200 μl of ice-cold 1.14 N perchloric acid containing 6 mM uranyl acetate was added to quench the reaction. The solution was kept in ice for another 30 min and centrifuged at 4 °C at 12000 rpm for 5 min. 100 μl of the supernatant was diluted to 1 ml and the change in absorbance measured at 260 nm and compared to a control set.

2.6. Inhibition kinetics

The inhibition of RNase A by the copper complexes of ECG and EGCG was assessed by a spectrophotometric method as described by Anderson et al. [22]. The assay was performed in oligo vinylsulphonic acid free 0.1 M Mes–NaOH buffer, pH 6.0, containing 0.1 M NaCl using 2',3'-cCMP as the substrate [25]. The inhibition kinetics for EGCG has been previously reported from this laboratory [8]. The substrate concentrations ranged from 0.04 to 0.09 mM and the inhibitor concentrations from 0 to 0.007 mM, with RNase A at 10 μM. The nature of inhibition and the inhibition constants was obtained from Lineweaver–Burk plots.

2.7. Fourier transformed infrared (FT-IR) studies

16.2 mg/ml (0.67 mM) RNase A was dissolved in 20 mM phosphate buffer of pD 7.2 in 99.9% D2O. RNase A:ligand ratios of 1:0.5 and 1:1.5 were prepared for the two complexes. FT-IR measurements were carried out at 25 °C on a Nexus-870 FT-IR spectrometer (Thermo Nicolet Corporation) equipped with a germanium-attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. Secondary structure information of uncomplexed and complexed RNase A were determined following the method of Bayler and Susi [26] as described elsewhere [27].
2.8. Circular dichroism (CD) measurements

CD measurements were made on a Jasco-810 automatic recording spectrophotometer, using 2 nm path length at 25°C. The RNase A concentration was 6.44 μM and complex mixtures with RNase A:ligand ratios of 1:0.5 and 1:1.5 were analyzed. Results were expressed as ellipticity (θ) and secondary structure determined using DICHROWEB [28].

3. Results and discussion

Reports on the ability of green tea polyphenols to inhibit angiogenesis in vivo [11] combined with promising results for copper reduction therapy [15] prompted us to probe the effect of copper complexes of two major green tea polyphenols on the enzymatic activity of RNase A. It is possible to extend these studies to proteins of the ribonuclease superfamily that exhibit unusual biological properties.

Spectrophotometric analysis on the complex formation of ECG and EGCG with Cu(II) indicated that with an increase in the concentration of Cu(II), the absorbance at 270 nm decreased with the formation of a broad shoulder at ~320–330 nm. Chelation studies of copper with flavonoid compounds such as quercetin, myricetin, catechin and luteolin report a similar spectroscopic shift [29]. The presence of two isobestic points at 263 and 290 nm in the spectra for Cu(II)-ECG and at 257 and 295 nm for Cu(II)-EGCG points toward the occurrence of two consecutive equilibria during complex formation (Fig. 2(a) and (b)). Jobs plots to determine the stoichiometry of the complexes are given in Fig. 2(c) and (d). The breakpoint at 0.3342 for ECG and 0.3247 for EGCG indicates that the stoichiometry of polyphenol to metal is 1:2 for both cases.

Electrophoresis experiments to check the effect on the ribonucleolytic activity of RNase A indicate that the complexes inhibit the enzymatic activity. The agarose gel based assay, monitored degradation of tRNA by RNase A and the effect of the complexes (Fig. 3(a)). The most intense band observed in lane 1 is of control tRNA. The faint band in lane 2 indicates the degradation of tRNA by RNase A. The differential intensity of bands in lanes 3, 4, 5, 6 qualitatively indicates the degree of RNase A inhibition by the Cu(II)-EGCG complex, Cu(II)-ECG complex, EGCG and ECG, respectively. Similar results obtained from the urea denaturing polyacrylamide gel electrophoresis are shown in Fig. 3(b). Control tRNA in lane 1 showed the least degradation; uncomplexed RNase A degraded tRNA to the highest extent in lane 2; reduced degradation patterns of tRNA are observed in lanes 3, 4, 5 and 6. These results show that Cu(II)-ECG and Cu(II)-EGCG act as effective inhibitors of RNase A.

A quantitative estimation of the relative inhibitory power of the polyphenols and their complexes is obtained from the precipitation assay. The plots of relative ribonucleolytic activity versus inhibitor concentration in Fig. 4 indicate that the Cu(II)-ECG complex is more effective compared to the Cu(II)-EGCG complex. It may be noted that the inhibitory efficacy of the copper complexes of the polyphenols is greater than the uncomplexed polyphenols. Kinetic experiments indicate that the two complexes behave as non-competitive inhibitors (Fig. 5). The inhibition constant values obtained for Cu(II)-ECG and Cu(II)-EGCG are 42 ± 1 and 23 ± 4 μM, respectively. Our previous study has shown that the inhibition constants for the inhibition of RNase A by EGCG and ECG were 81 μM [8] and 392 ± 23 μM [30], respectively. The inhibition constant values obtained for Cu(II)-ECG and Cu(II)-EGCG correlate well with the results from the precipitation assay with a correlation coefficient of 0.93.

Protein amide I bands at 1645–1650 cm⁻¹ (mainly C=O stretching) and the amide II band at 1548–1560 cm⁻¹ (C–N stretching coupled with N–H bending mode) [26] change upon ligand binding. Hydrogen bonding and the coupling
between transition dipoles play a crucial role in governing the conformational sensitivity of the amide bands of the protein. Spectral ranges from 1610 to 1632 cm\(^{-1}\), 1636 to 1644 cm\(^{-1}\), 1650 to 1662 cm\(^{-1}\) and 1665 to 1680 cm\(^{-1}\) in the amide I region are attributed to \(\beta\)-sheet, random coil, \(\alpha\)-helix and turn structures, respectively [31,32]. The peak positions of the amide I and II bands in the two difference spectra of each complex shift with a simultaneous change in the relative intensity (Fig. 6), indicating a change in the secondary structure of the protein. In the difference spectra of Cu(II)-ECG at a protein ligand ratio of 1:1.5, the peak maximum at 1643 cm\(^{-1}\) with a shoulder at 1660 cm\(^{-1}\) means that there are changes in the \(\alpha\)-helical structure. In case of Cu(II)-EGCG at a protein ligand ratio of 1:1.5, changes in the peak and shoulder positions reflect the changes in \(\alpha\)-helix content (1662 cm\(^{-1}\)) and random structure (1644 cm\(^{-1}\)). The increase in intensity observed in the difference spectra between 1546 and 1552 cm\(^{-1}\) for the two complexes can be attributed to the interaction of the polyphenols with the backbone of the protein. A quantitative analysis of the secondary structural elements is given in Table 1.

CD spectroscopic studies of free RNase A and with Cu(II)-ECG and Cu(II)-EGCG also indicate a perturbation of the secondary structure (Fig. 7). The effect on the secondary structure is more pronounced for Cu(II)-EGCG. Secondary structure content was determined using DICHROWEB for RNase A polyphenol complexes of 1:0.5 and 1:1.5 ratios and compared with FT-IR results (Table 1). We observe that for both FT-IR and CD studies there is an increase in the \(\alpha\)-helix content (8–9%) with a concomitant reduction in random structure. The increase in \(\alpha\)-helix content of RNase A has also been observed with 3' azido-3' deoxythymidine (AZT) [33].

This study suggests that the copper complexes are more potent inhibitors of RNase A than the parent polyphenols. Based on the structural homology between RNase A and angiogenin these complexes can subsequently be used for antiangiogenic therapy through copper chelation. Further studies are underway to ascertain the effect of the complexes on the angiogenic activity of angiogenin since the enzymatic and biological activities of this protein are related. These studies also provide a guideline as to how antangiogenic compounds based on these complexes may be developed to limit undesirable vascular proliferation.

Fig. 5. Lineweaver–Burk plots for inhibition of RNase A by (a) Cu(II)-ECG and (b) Cu(II)-EGCG. Concentrations: 0.0067 mM (■), 0.004 mM (▲), 0 mM (●).

Fig. 6. FT-IR difference spectra of RNase A complexes with (a) Cu(II)-ECG and (b) Cu(II)-EGCG at two different concentrations, RNase A:polyphenol 1:0.5 (●●●) and 1:1.5 (●●) (left-hand axis) with FT-IR spectra of free RNase A (●) (right-hand axis).
Acknowledgements: S.D.G. is grateful to Council of Scientific and Industrial Research (CSIR), India for financial support. T.K.M. thanks CSIR, New Delhi for a fellowship. The authors are grateful to Dr. Munna Sarkar for helpful discussion.

References


Table 1

<table>
<thead>
<tr>
<th></th>
<th>α-Helix (%)</th>
<th>β-Sheet (%)</th>
<th>Random coil (%)</th>
<th>Turn (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FT-IR</td>
<td>CD</td>
<td>FT-IR</td>
<td>CD</td>
</tr>
<tr>
<td>RNase A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.0</td>
<td>15.0</td>
<td>38.0</td>
<td>42.0</td>
</tr>
<tr>
<td>RNase A:Cu(II)-ECG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:0.5</td>
<td>19.0</td>
<td>14.0</td>
<td>45.0</td>
<td>43.0</td>
</tr>
<tr>
<td>1:1.5</td>
<td>18.0</td>
<td>14.0</td>
<td>38.0</td>
<td>44.0</td>
</tr>
<tr>
<td>RNase A:Cu(II)-EGCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:0.5</td>
<td>20.0</td>
<td>15.0</td>
<td>38.0</td>
<td>44.0</td>
</tr>
<tr>
<td>1:1.5</td>
<td>31.0</td>
<td>23.0</td>
<td>38.0</td>
<td>44.0</td>
</tr>
</tbody>
</table>

Fig. 7. CD spectra of (a) RNase A-Cu(II)-ECG and (b) RNase A-Cu(II)-EGCG complexes. Free RNase A (–), RNase A:complex 1:0.5 (–→–) and RNase A:complex 1:1.5 (–△–).


