Reaction between Ellagic Acid and an Ultimate Carcinogen

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The reaction coordinate between a typical ultimate carcinogen benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) and ellagic acid, a proven chemopreventive agent active against cancers caused by polycyclic aromatic hydrocarbons (PAHs), was examined by density functional theory (DFT) and semiempirical MO calculations, and activation energy was calculated. The effect of a polar environment was included using Tomasi and the Langevin dipoles methods. The calculated BPDE/ellagic acid reaction free energy of activation is found to be in decent agreement with experimental data [Sayer, J. M. et al. *J. Am. Chem. Soc.* **1982**, *104*, 5562−5564]. This work sheds light on the mechanism of action of ellagic acid. Quantum chemical calculations of this kind are valuable for the design of ellagic acid derivatives with even lower activation energy and increased reactivity toward ultimate carcinogens as well as controlled reactivity toward DNA.

INTRODUCTION

Cancer is a major cause of death in developed countries, second after cardiac disease. Chemical carcinogens are being implicated in the aetiology of an increasing number of cancers. Examples include bladder (hair dyes[1,2]), lung (smoke chemicals, reviewed by Hecht, 1999[3]), and hepatocellular (aflatoxins, reviewed by Guyton and Kensler, 2002[4]) carcinomas. An extensively studied and very potent group of chemical carcinogens are the polycyclic aromatic hydrocarbons (PAHs) (refs 5 and 6: reviews, and ref 7). PAHs are prevalent environmental contaminants, formed in the combustion of organic substances. Diesel engine exhausts and cigarette smoke contain a large number of different PAHs, e.g. benzo[*a*]pyrene, benz[*a*]anthracene, or dibenz[*a*,*h*]anthracene.

The link between PAHs and cancer was recognized over 200 years ago. PAHs per se are not carcinogenic but metabolites of these molecules, called ultimate carcinogens.[8−10] These ultimate carcinogens are benzo-ring diol epoxides in which the epoxide group is located besides the bay region of the molecule. A typical example of a PAH, benzo[*a*]-pyrene (B[*a*]P), is activated by cytochrome P450 to produce the ultimate carcinogen benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE).[11−13] Diol epoxides exist as pairs of diastereomers in which the hydroxyl groups and the epoxide oxygen are either cis or trans one with regard to the others. In the case of B[*a*]P, only the (+)-enantiomer of the cis-8-hydroxy-9,10-epoxy isomer with trans 7 and 8 hydroxyls is highly tumorigenic.[14−16]

Carcinogenic activity of the diol epoxides is due to alkylation of DNA at the angular bay region of the PAH.[17] The exocyclic amino groups of the purine nucleosides, deoxyguanosine and deoxyadenosine, are the major sites of alkylation,[18] although adducts at other positions are possible, e.g. N7 of guanine or 2′-hydroxyl group of the sugar.[19] Adducts are formed by nucleophilic attack at C10 of BPDE, opening the epoxide ring with stereochemical inversion to yield the trans product. BPDE-DNA adducts create 'hot spots' in the p53 tumor suppressor gene, inducing p53 mutations which will favor several types of cancer, e.g. human lung cancer,[20] stomach, breast, colon, prostate, or brain cancers.[21]

While the pathogenesis of cancer is complex and multifactorial, a common observation is that carcinogenesis may be initiated by an adduct formation between an ultimate carcinogen and DNA, followed by mutation or DNA damage.[22,23] Studies indicate a dose dependent relationship between the amount of carcinogen and the level of adducts, and adduct formation can be considered as a marker for exposure to and carcinogenicity of a chemical. Indeed there is evidence that adducts can modulate enzymes such as topoisomerase I[24] and lead directly to alterations in the expression of genes involved in carcinogenesis.[25] DNA repair mechanisms exist to reverse DNA damage, for example nucleotide excision repair for the replacement of BPDE-DNA adducts by the correct base or base dimer. However these mechanisms can fail or become overwhelmed; adduct formation can itself modulate DNA repair genes.[25] It would therefore be highly desirable to be able to prevent the initial step of carcinogens chemical binding to DNA, with the use of chemopreventive agents competing with the carcinogens.

Ellagic acid (EA), a naturally occurring plant polyphenol found particularly in red raspberries, is capable of chemically reacting with the B[*a*]P metabolites and preventing the covalent binding of BPDE to DNA.[26,27] Many studies have confirmed the cancer chemopreventive properties of berries.[28,29] Ellagic acid has indeed been shown to inhibit

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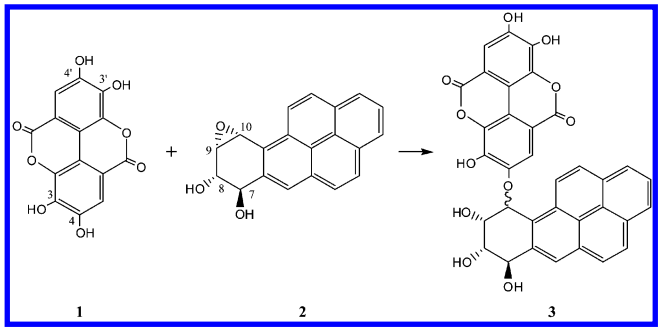

Figure 1. Reaction between ellagic acid (**1**) and BPDE (**2**) to lead cis- or trans-products (**3**).

carcinogenesis in both in vitro assays[30,31] and in vivo studies, e.g. of chemically induced cancer in the lung, liver, skin, and esophagus of rodents or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in mouse skin (refs 32−35, reviewed by Stoner and Mukhtar, 1995[36]).

The mechanisms by which ellagic acid inhibits mutagenesis and carcinogenesis are not entirely clear. Inhibition by ellagic acid of DNA adduct formation with PAH metabolites has been demonstrated in cell free microsomal assays,[37] mouse lung cultures,[30] and mammary tumor cell lines,[38] although this phenomenon could not be observed in all experimental studies.[39,40] A direct effect on scavenging and detoxification of the diol epoxide of B[*a*]P as proposed by Jerina and co-workers is supported by several in vitro studies[30,41,42] and suggested in in vivo studies by Chang et al., who found ellagic acid to be more effective against BPDE (in this case the 9,10-epoxide-2 form) than B[*a*]P experimentally induced tumorigenesis.[43]

Ellagic acid also inhibits metabolic activation of PAHs as well as nitroso compounds and aflatoxins. It has been reported to inhibit the CYP1A1-dependent activation of B[*a*]P,[44] to reduce the formation of O6-methylguanine by methylating carcinogens in binding itself to DNA,[45,46] and to induce phase II detoxification enzymes glutathione S-transferase Ya and NAD(P)H:quinone reductase.[47] In addition, ellagic acid has strong antioxidant activity[48] and may play a role in the regulation of the cell cycle in cancer cells.[49]

Ellagic acid acts as a scavenger by chemical binding of the ultimate carcinogens. In this study quantum chemical methods were used to calculate the free energy of activation for the reaction between ellagic acid (**1**) with the ultimate carcinogen (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (**2**) (Figure 1). According to Jerina and co-workers[26] the reaction of **1** with **2** is believed to be of the S_N1 type. The phenolic OH group approaches the epoxy ring carbon atom. During the reaction the strained epoxy ring opens, and formation of the chemical bond between the epoxy ring carbon atom and the phenolic oxygen atom is preceded by proton transfer. Proton transfer formally ensures carbocation as an intermediate. In the case of strict separation of these two events the reaction would be of the pure S_N1 type and both cis- and trans-isomers (**3**) on the epoxy carbon atom would be equally observed. In the present case these events are not strictly separated, and experiments reveal that the reaction ends with products that are of neither inverted chirality (S_N2) nor present in a racemic mixture (S_N1). Indeed, the carbocation may also form at position 9, but as this does not lead to the major product, this ion must be less stable. It is also believed that in the transition state,

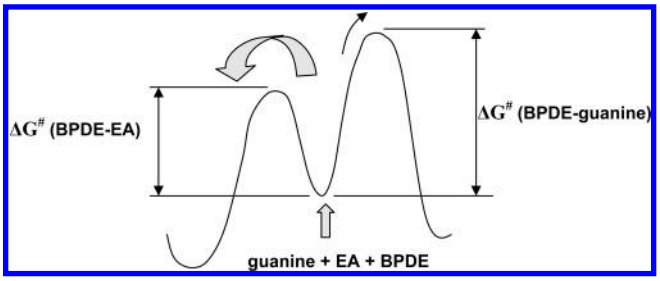

Figure 2. Likely scheme of a favored reaction of the ultimate carcinogen BPDE with ellagic acid, compared to guanine.

a possible adduct could be formed through $\pi-\pi$ stacking and hydrogen bonding between EA and BPDE, orienting EA well for a cis nucleophilic attack on C10, which would favor the formation of this isomer. These products are capable of further rapid reaction with **2**, by attack of the opposite phenolic hydroxyl of EA moiety. Here we have chosen the C−O distance as the reaction coordinate and optimized all other degrees of freedom. With this approach we avoided arbitrary choice of the reaction mechanism. Experimental data show that the rate constant is very much pH dependent. Ellagic acid may react with **2** in the neutral, monoanionic, and dianionic forms. Since the major contribution to the rate constant at physiological pH originates from the neutral form, we only considered this reaction channel.[26]

In this work we want to verify the proposed mechanism of Jerina and co-workers, who found experimentally the activation energy between BPDE and ellagic acid to be pH dependent and at the physiological pH (at 25 °C) to have a value of 20.7 ± 0.2 kcal·mol^{-1}.[26]

Free energy of activation of the reaction between ellagic acid and BPDE was calculated using semiempirical MO and DFT methods, while the effect of hydration was considered using Tomasi and Langevin dipoles methods. There is a one-to-one correspondence between the free energy of activation and the rate constant.[50] If the activation free energy for the reaction between an ultimate carcinogen and ellagic acid is lower than the corresponding reaction with e.g. guanine, this therefore means that the former reaction will be faster. The equilibrium will thus be displaced toward the formation of the ultimate carcinogen/EA adduct rather than the one with guanine in DNA (Figure 2). The reaction of alkylation of DNA by BPDE is very difficult to apprehend; indeed, a thorough study by Geacintov et al.[51] demonstrates that several parallel reactions are possible, including hydrolysis of free BPDE, DNA catalyzed hydrolysis, and noncovalent DNA intercalation of BPDE. In the scheme of Figure 2 activation energy for the reaction between EA and guanine would of course also have to be considered.

To the best of our knowledge this is the first computational study aiming at evaluating chemical reactivity for the reaction between the ultimate carcinogen BPDE and the scavenger polyphenol ellagic acid.

COMPUTATIONAL METHODS

DFT and semiempirical calculations were performed to examine the reaction coordinate for the reaction between ellagic acid and the epoxidized aromatic compound. The distance between the carbon atom of the epoxy ring C10 of BPDE and the enol oxygen linked to C4 of EA (see Figure 1) was chosen to be the reaction coordinate. First the minima

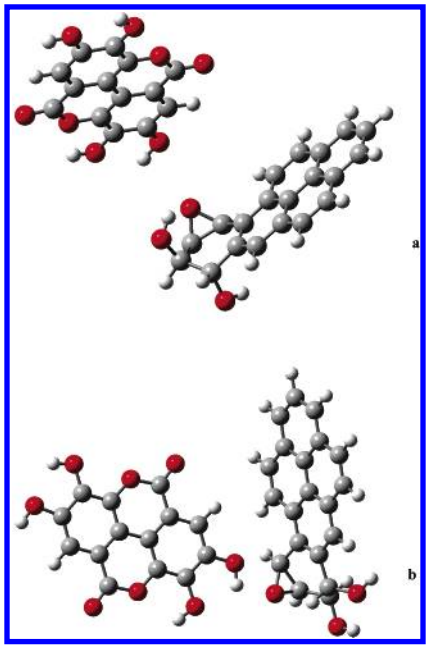

Figure 3. Reactants (a) and transition state (b) (image generated with GaussView). Left: ellagic acid, right: BPDE. Minimized structures. Dark gray: carbons, light gray: hydrogens, and red: oxygens.

corresponding to reactants and products were calculated. Minima were proved to be the real minima rather than transition states by performing vibrational analysis in the harmonic approximation (*vide infra*). Our first trial to locate the transition state automatically using the methods implemented in Gaussian-98 failed. Therefore the transition state was calculated by varying the reaction coordinate between the minimized reactants and products in a systematic way (Figure 3).

The search was performed by minimization of energy with respect to all degrees of freedom but the fixed value of the reaction coordinate. The so obtained energy profile corresponds to the minimum energy path. Vibrational analysis in the harmonic approximation was performed for the structures corresponding to the transition state and the reactants. For the reactants all frequencies were real, while the transition state had one imaginary frequency. For the points belonging to the product valley we have rewritten the Z-matrix such that the migratory proton was bonded to the epoxy oxygen atom.

Density functional theory (DFT) calculations were performed at the B3LYP level using the basis set 6-31G(d). This double-ζ basis set augmented with polarization functions on heavy atoms is flexible enough to faithfully describe the interaction energy of molecular complexes of moderate strength and is still computationally tractable. The applied DFT method includes the calculation of an exchange functional by the method proposed by Becke[52] and a correlation functional suggested by Lee, Yang, and Parr.[53] B3LYP calculated energies thus contain a significant part of the correlation energy. The semiempirical MO method PM3 was examined since due to the low CPU cost it is suitable for QM/MM calculations, which include thermal averaging, for such kinds of reactions. Indeed the PM3 method shows in general remarkably good agreement with DFT results, for many systems of biological interest, which was verified in our present study. For instance it gives a reliable Born−Oppenheimer hypersurface in the study of hydride transfer catalyzed by xylose isomerase.[54]

The solvent reaction field of Tomasi and co-workers[55,56] was applied on the B3LYP/6-31G(d) level to calculate the free energy of hydration for reactants and the transition state. The Langevin dipoles (LD) method parametrized by Florian and Warshel[57] was applied to calculate the free energies of hydration for the reactants and the transition state, that when combined with in vacuo activation energy gives rise to a total free energy of activation. There is a one-to-one correspondence between the free energy of activation $\Delta G^{\#}$ for a chemical reaction and the rate constant k of this reaction

$$k = \frac{k_B T}{h} \cdot e^{-\Delta G^{\#}/k_B T}$$

where k_B is the Boltzmann's constant, T is the absolute temperature, and h is Planck's constant. PM3 and DFT calculations as well as Tomasi's free energies of hydration were performed using a Gaussian-98 suite of programs.[58] The Tomasi values were compared to the ones obtained using the LD program ChemSol 2.1,[59] kindly provided by Jan Florian. In LD calculations Merz−Kollman atomic charges were determined on a B3LYP/6-31G(d) level with an included solvent reaction field by the method of Tomasi, using B3LYP/6-31G(d) minimized geometries for reactants and the transition state. A closely related approach was used to calculate the activation energy for the irreversible inhibition of HIV-1 protease.[60]

All the programs were implemented on a cluster of 64 PC/Linux based machines running AMD Athlon processors at 700 MHz.

RESULTS AND DISCUSSION

The calculated activation free energy for the chemical reaction between (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene and the polyphenol ellagic acid was found to be 26.9 kcal·mol^{-1}. This value includes the B3LYP/6-31G(d) calculated classical barrier, 1.40 kcal·mol^{-1} of B3LYP/6-31G(d) calculated zero point energy correction and 1.26 kcal·mol^{-1} of difference in free energy of hydration calculated using the Langevin dipoles method version 2.1. In comparison, Tomasi's approach for hydration free energy difference gave a value of 3.15 kcal·mol^{-1}. The corresponding PM3 calculated classical barrier was 25.7 kcal·mol^{-1}. The reaction pathway is shown in Figure 4, where energy is plotted as a function of the reaction coordinate.

The calculated value for the activation energy is in fair agreement with the experimentally determined value of 20.7 $\pm$ 0.2 kcal·mol^{-1} at a physiological pH of 7.4 and 25 °C (or 20.5 at pH 7).[26] The agreement between the calculated and the experimental value is not perfect. One possible source of discrepancy could be the dioxane/water mixture used to determine the experimental rate constant. The experimental data show that changing the dioxane concentration from 10% to 0.3% at pH 7.4 speeds up the reaction by a factor of 12, which would decrease the value of activation energy for about 7%. Our mean field treatment of solvent cannot properly describe such effects. The applied level of theory is a compromise between the reliability of the results and available CPU power. The studied system is large and

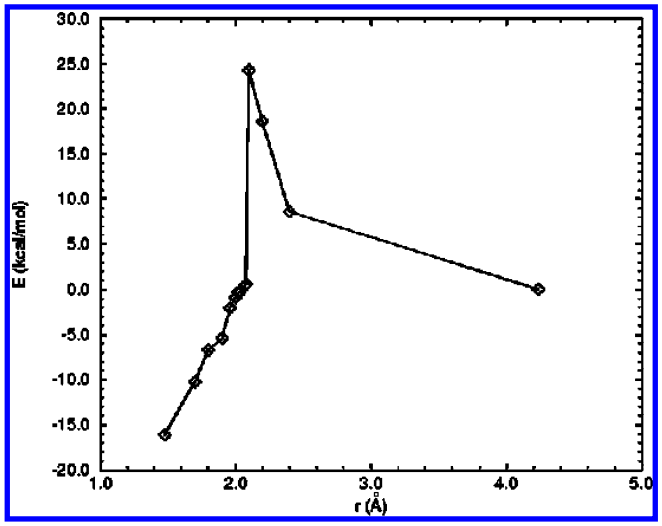

Figure 4. B3LYP/6-31G(d) calculated energy (in kcal·mol^{-1}) as a function of the reaction coordinate that is defined as the distance between C10 of BPDE and the enol oxygen on C4 of ellagic acid. All energies are relative to the reactants. Please note that the two extremal points in the diagram belong to the products (1.476 Å) and reactants (4.237 Å) and were obtained by full energy minimization.

relatively flexible, and therefore the location of the transition state required several hundreds of evaluations of the forces and energies. Given the too large barrier calculated on a B3LYP level we tried to perform also MP2/6-31G(d) calculations. We nevertheless did not manage to perform these calculations, despite the fact that we tried on several platforms with up to 4GB RAM. More reliable treatment of the environment (e.g. explicit inclusion of water molecules) would be beneficial. Indeed a more protic solvent would increase the rate of the reaction by favoring the ring opening of the epoxide, which is supported, as already mentioned, by the experimental finding that decreasing the dioxane concentration from 10% to 0.3% increases the reaction rate substantially. To check a possible effect, we included two explicit water molecules around the epoxide ring and ran the calculations on the PM3 level. This system was extremely floppy, and several hundred geometry optimization steps were necessary. The barrier was lowered to 21.4 kcal·mol^{-1}, what is in almost perfect agreement with the experimental value. This agreement is yet most probably fortuitous, and thermal averaging of solvent degrees of freedom by molecular dynamics as well as for ellagic acid and the ultimate carcinogen would be necessary. The methods are developed (QM/MM) and ready to be used. This would notably allow for checking the hypothesis of Jerina et al. that the exceptional reactivity between EA and BPDE is caused by specific interactions between their aromatic ring systems. Our optimizations of the transition states did not end up with such stacking (see Figure 3); however, the hydrophobic effect could well lead to this configuration. We believe that application of the advanced post Hartree−Fock level of theory together with a better treatment of the environment would significantly improve our grip of the physics of the system and agreement with experimental data. In particular, calculation of the potential of mean force for this reaction using QM/MM or Car-Parrinello approaches remains a challenge for the future.

This study supports that detoxification of BPDE by EA is a likely mechanism by which EA could prevent cancer. EA has been shown to be chemopreventive in several animal models of carcinogenesis. We have only studied one aspect of EA preventive action, but antioxidant effects of EA on the inhibition of enzymes involved in the metabolism of xenobiotics and carcinogens have also been demonstrated.[42,61] Nevertheless inhibition of PAH-DNA adduction by EA seems to be entirely or in part enzyme independent since it has been shown that EA inhibits BPDE (or diBPDE)-DNA adduction in the absence of enzymatic influences.[37] EA may also directly react with guanine, acting to prevent DNA alkylation. However in this situation it is worth noting that the affinity of EA for DNA is five times greater than for proteins,[62] and it is therefore critical to know the concentration at which EA becomes carcinogenic itself. In some studies a chemopreventive effect has not been observed or observed only at a toxic dose of ellagic acid. This could also be related to its bioavailability, as has been shown in studies on several animal models.[62−64] In humans, bioavailability of EA from dietary sources seems to have been confirmed only with red raspberries. The inhibition of DNA-BPDE adduct formation by EA in an organism is thus dose-dependent, and the therapeutic-to-toxic ratio is narrow. It is not clear how cytosine methylation (e.g. in certain codons of the P53 gene), which can enhance adduct formation,[65] intervenes in the mechanism of action of EA. Moreover EA derivatives or analogues are themselves able to bind to DNA, with potentially adverse consequences.[66] Clearly, a greater understanding of EA chemistry and biology is required in order to optimize its cancer preventive properties. Finally it is of note that EA has little effect on established tumors.

Studies by Barch et al.[42] demonstrated that different putative portions of the ellagic acid molecule may be indispensable for its anticarcinogenic activities. Both the 3-hydroxyl and the 4-hydroxyl groups are required for EA to directly detoxify the diol epoxide of benzo[*a*]pyrene, while only the 4-hydroxyl group is necessary for inhibition of CYP1A1-dependent benzo[*a*]pyrene hydroxylase activity. This in general includes blocking of phase I enzymes involved in the metabolic activation of carcinogens or induction of phase II enzymes involved in detoxification of reactive species.

Many naturally occurring phytochemicals are being investigated for their chemopreventive anticancer potential. Examples include sulforaphane, the principal phase II gene inducer in broccoli, or indole-3-carbinol (glucosinolate hydrolysis products) (refs 67−69, reviews), the most abundant and popular molecule studied in the research of effective tea polyphenols (−)-epigallocatechin-3-gallate, which acts as a powerful antioxidant and can inhibit a number of tumor cell proliferation- and survival-related proteins (ref 70, review), and finally, the series of polyphenols isolated from Blumea balsamifera, which seems to be very promising for the prevention of various carcinoma.[71]

CONCLUSION

Our study demonstrates that it is possible to calculate the activation free energy between a typical ultimate carcinogen, here benzo[*a*]pyrene-7,8-diol-9,10-epoxide, and ellagic acid. The fairly good correspondence between our computed value and the value that can be determined from Jerina and coworkers experiments[26] is strong evidence that the proposed

reaction mechanism approach is valid. It is likely that prevention of DNA adduct formation by direct scavenging of the carcinogen is an important mechanism of EA action. EA therefore belongs to a category of agents that hit cancer at many different levels—enhancing detoxifying enzymes, modulating cancer cell cycle, acting as an antioxidant—and is also able to quench an ultimate carcinogen directly prior to reaching its DNA target.

Yet because of its toxicity at high concentrations, ellagic acid is not an ideal drug. This will have to be bypassed by designing novel ellagic acid derivatives that will also take into account their bioavailability and pharmacokinetics. When performing this study we had in mind the use of EA as a lead compound that can be optimized, i.e., modified in a way to give rise to compounds with even lower activation free energy, with enhanced detoxifying ability and without undesirable reactions with other biological molecules, including DNA. Different combinatorial chemistry methods have been developed[72,73] in order to achieve this aim. When completed by computational support, these could be readily implemented in the design of novel chemopreventive drugs.[74]

In conclusion, we believe that there is still plenty of space for development of ellagic acid analogues with favorable activation energy for the reaction with ultimate carcinogens and high activation energy for the reaction with DNA. Our study demonstrates that the reactivity of any cancer preventing agent may be analyzed computationally at the drug design stage. We believe that calculations of this type will serve as a computational tool for the design of novel and even more reactive polyphenols that could be used for cancer prevention.

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