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Characterization of folic acid/native cyclodextrins host–guest complexes in solution

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ABSTRACT

The complexation of folic acid (FA) with native cyclodextrins was studied and this process was used for the comparison of ^{1}H NMR, ITC and ESIMS for the evaluation of association constants. The stability increases in the series: α-cyclodextrin/FA < γ-cyclodextrin/FA < β-cyclodextrin/FA. ^{1}H NMR and ITC gave comparable results in regard to association constant values, while results obtained for MS were considerably higher due to different interactions (electrostatic instead of hydrophobic) responsible for the stabilization of the complexes. The dimerization of FA in water was also studied, as well as its impact on the process of complexation with native cyclodextrins.

1. Introduction

Synthetic folic acid (FA, Fig. 1), the fully oxidized form of tetrahydrofolate, belongs to the group of water-soluble vitamins B_9. Tetrahydrofolate and its derivatives take part in many biological processes in the human body. They play important role in some cellular pathways, where folate acts as one-carbon source – DNA synthesis, methylation and sustenance, as well as RNA and protein methylation. They are required for the process of biosynthesis of proteins and nucleic acids [1]. Folate receptor is overexpressed in many tumors, such as brain, breast, kidney and lung cancers [2]. The density of the folate receptor grows significantly in the later stages of cancer and, in the case of classical treatment methods failing, folate-derived therapeutics may be in use. Prodrugs are usually conjugates of FA with various drugs, such as cytotoxic drugs, e.g. doxorubicin [3] camptothecin [4] and taxol [5]. Due to the variety of possible pharmaceutical applications it is important to improve bioavailability and aqueous solubility of FA and its derivatives (e.g. various folate-based prodrugs).

Native cyclodextrins (CDs) – α, β and γ (Fig. 1) – are macrocyclic oligosaccharides built from 6, 7 or 8 glucose units linked by α-1,4-glycosidic bonds. They possess hydrophobic cavity of different radius and sizes, which is able to accommodate various molecules and hydrophilic outer face, which makes them easily soluble in water. They improve the solubility, stability, and bioavailability of biologically active compounds. Due to their specific properties they are used by cosmetic [6], food [7] and pharmaceutical industries [8,9].

Recently, we have studied the formation of host–guest systems between native CDs and FA [10]. The analysis of ^{1}H NMR and 2D NMR spectra led to the conclusion that CDs characterized by larger cavity volume – β- and γ-CDs form with FA rotaxane-like structures, while the smallest α-CD forms an exclusion compound. According to the preliminary results obtained from Mass Spectrometry experiments the following series of complexes' stabilities in the gas phase was postulated: γ-CD/FA > β-CD/FA > α-CD/FA. These results were further confirmed by Ion-Mobility Mass Spectrometry and theoretical calculations [11]. The aim of the work presented here is to study in detail and to compare stabilities of the obtained host–guest systems in solution with the previously obtained results for the gas phase. Techniques involved in the study

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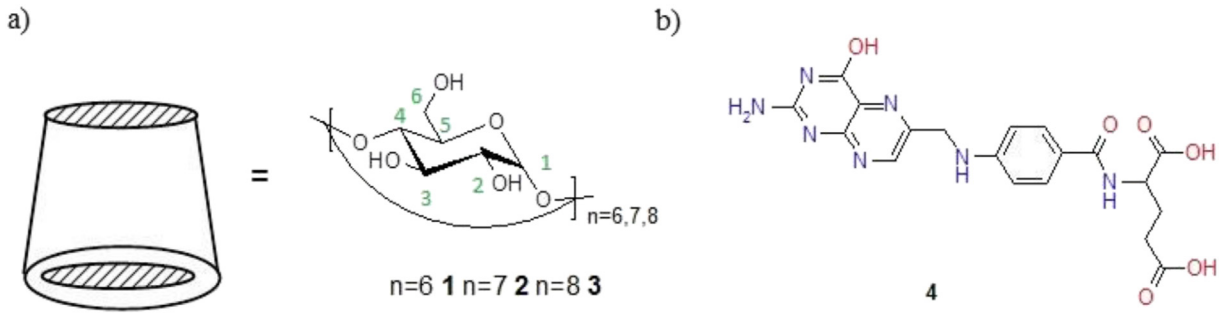

Fig. 1. Schematic presentation of a) host molecules – α, β and γ-CDs (**1**–**3**) and b) guest – folic acid (**4**), numbering of cyclodextrin carbon atoms in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

include ^{1}H NMR, Isothermal Titration Calorimetry and HPLC-MS. ^{1}H NMR allowed for determination of association constants of α-CD/FA, β-CD/FA and γ-CD/FA in water, it also enabled the study of FA dimerization in water. Isothermal Titration Calorimetry allowed for the confirmation of the results obtained from ^{1}H NMR studies, giving also the answer of the association constant of the strongest of the studied complexes in physiological buffer (HEPES). MS method was also applied to determine the association constants of α-CD/FA, β-CD/FA and γ-CD/FA complexes. In those measurements mass spectrometer was treated as detector for quantitative analysis of CD/FA complexes. In contrast to the previously obtained gas-phase stability order [10] in this approach the solution-phase binding details such as association constants and binding stoichiometry may be obtained under certain conditions. For example, it is very important to carefully set up the experimental parameters to avoid dissociation of the complex or change of the solution-phase equilibria while transferring ions to the gas phase. The type of interactions occurring within complex affects significantly the agreement between solution- and gas-phase association constants, since each type of interactions responds differently on the transition from solution to the gas phase. The stabilization from hydrophobic interactions which play a leading role in cyclodextrin inclusion complexes is significantly diminished in the gas phase, while electrostatic interactions are supposed to be strengthened in vacuum. In this study we evaluate the MS approach to determine the solution association constants of FA/CDs complexes on the basis of the comparison with other methods as well as with the pure gas-phase stabilization results.

2. Experimental

2.1. Materials

Folic acid was commercially available product (obtained from Sigma Aldrich, Germany) and was in a form of sodium salt. α, β and γ cyclodextrins were obtained from Cyclolab (Hungary) and used as received. HEPES was obtained from Roth. Distilled water was used for aqueous solutions. D_2O (99.9% D) was purchased from Cambridge Isotope Laboratories.

2.2. Methods

2.2.1. Nuclear Magnetic Resonance (^{1}H NMR)

The ^{1}H spectra were recorded on Varian Mercury 400. All measurements were performed in D_2O. Chemical shifts are reported in ppm. The splitting pattern of multiplets is described by abbreviations (*s* – singlet, *d* – doublet, *dd* – doublet of doublets, *m* – complex multiplicity).

2.2.1.1. Dimerization of folic acid sodium salt in water. D_2O was titrated in an NMR tube with the 0.22 M D_2O solution of FA sodium salt until the concentration reached c = 0.038 [M]. Dimerization constant was evaluated using HypNMR program [12,13].

2.2.1.2. Titration studies for evaluation of stability constants. The ca. 0.01 M D_2O solution of a receptor (α, β and γ-CD, respectively) was titrated in an NMR tube with the 0.25 M solution of a FA sodium salt. The solution of the salt contained a certain amount of the proper CD in order to keep its concentration constant during the titration. For each titration 15 data points were recorded. The corresponding association constants K_as were calculated using non-linear regression algorithm on the basis of the all chemical shift changes by the HypNMR 2008 software [12,13]. A nonlinear curve fitting for 1:1 binding model was carried out with the HypNMR program.

2.2.2. Isothermal Titration Calorimetry

ITC measurements were carried out at 298.15 K on a Microcal OMEGA ultrasensitive titration calorimeter (MicroCal Inc.). A proper cyclodextrin and FA sodium salt were dissolved in water and degassed prior to measurements. For β-CD additional experiments in 0.1 M Hepes buffer, pH 7.40 were performed. The solutions in the cell were stirred at 400 rpm. Equal volumes of FA sodium salt solutions were injected into the sample cell containing α-CD/β- CD/γ-CD over 20 s with an interval of 170 s between injections from a 250 μl injection syringe, in a series of controlled pulses. The sample cell volume was 1.3611 cm^3. The integrated heat effects of each injection were corrected by subtraction of the corresponding integrated heat effects of folic acid salt injections into the pure buffer and water and heat effects of buffer/water injections into the CD solutions. Standard deviation was calculated by the model of a single set of identical sites (ITC Tutorial Guide).

2.2.3. Mass spectrometry measurements

MS measurements were carried out using a *4000 Q TRAP* (*Applied Biosystems Inc, USA*), equipped with an electrospray (ESI) ion source (*TurboIonSpray*), the triple quadrupole/linear ion trap mass analyzer and coupled to High-Performance Liquid Chromatograph *Prominence LC-20* (*Shimadzu*). The analysis was carried out in the negative ion mode. The ion source parameters were tuned for the maximum response of the noncovalent complexes of $[CDs/FA - 2H]^{2-}$ and were set at the following values: ion spray voltage (IS) 4500 V, declustering potential (DP) 50 V, entrance potential (EP) 14 V, ion source temperature 300 K. Samples were infused into the mass spectrometer at injection volume of 10 μl using HPLC system through autosampler with the flow rate of 0.2 ml/min, H_2O:MeOH (1:1) of mobile phase. The spectra were recorded in the MRM ion mode at unit mass resolution. The