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Thermodynamics of complexes formation by ITC in methanol/water = 9/1 (v/v) solution: A case study

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ABSTRACT

Most enzymes that participate in the biochemistry of nucleic acids require divalent metal ion cofactors to promote activity. Development of potent inhibitors, acting against those viral enzymes operating via a cooperative two-metal ion mechanism, such as HIV integrase (IN) and RNase H, hepatitis C virus polymerase and influenza endonuclease, requires optimizing the binding affinity to the target, which is dictated by the binding free energy composed of both enthalpic and entropic contributions. They can be obtained by using isothermal titration microcalorimetry. We have defined an experimental procedure for obtaining reliable thermodynamic data in methanol/water = 9/1 0.1 M KCl as solvent, used to overcome solubility problems. In this way we have measured the heats of formation of the complexes formed by N-(4-fluorobenzyl)-5-hydroxy-2-isopropyl-1-methyl-6-oxo-1,6-dihydroxypyrimidine-4-carboxylate (HL, a model of Raltegravir, the antiretroviral drug produced by Merck & Co.), and a series of divalent metal ions of biological interest (Mg(II), Mn(II), Co(II) and Zn(II)), whose speciation was previously determined by potentiometry.

1. Introduction

Many metal ions play a vital role in biological functions in humans (e.g. sodium, potassium, magnesium, calcium, iron, zinc, copper, manganese, chromium, molybdenum and selenium) and currently many metal-based compounds or metal salts are routinely administered to patients for therapeutic and diagnostic purposes. Essential metal nutrients are supplied in supplemental form to overcome deficiencies, or metals are removed via chelation in the case of overload. From the discovery of cisplatin, still used against specific types of cancer, a great variety of metal-based anticancer drugs has been designed and studied [1]. Gold complexes have been extensively investigated as antibiotic, antiarthritic and anti-cancer agents, bismuth complexes as potent metalloenzyme inhibitors, anti-diarrheals and anti-bacterials, ruthenium complexes have been shown to exhibit highly encouraging anti-cancer activity, vanadium sulphate is being investigated in diabetes research as a potential alternative to insulin in type II diabetes ([1–5] and references therein). More recently, metal coordination complexes have been employed in molecular recognition of biological substrates [6], specifically as protein biosensors and as inhibitors of protein complexation events and of biological functions. Moreover, many molecules carry out their therapeutic action because they are able to bind the metal in the metallo-enzyme active site. Magnesium ion, for instance, is an essential cofactor in numerous enzymes such as polymerases, exonucleases, ribonucleases, transposases, and integrases and in many processes involving formation and modification of phosphate chains. Understanding the mechanism of action of the inhibitors that act by chelating magnesium is therefore of great importance in order to develop new antiviral drugs [7]. Development of potent inhibitors requires optimizing the binding affinity to the target, which is dictated by the binding free energy composed of both enthalpic and entropic contributions. Structure-based drug design enormously benefits from thermodynamic profiles, which provide information on the driving forces for binding. For some years we are studying new HIV-1 integrase inhibitors [7–10]. HIV-1 integrase (IN) catalyzes the integration of proviral cDNA into the host cell genome; it is an essential enzyme for viral replication [7–13] and a validated target for the development of drugs against AIDS [14–16]. Several IN inhibitors were identified through in vitro inhibition assays with recombinant IN [17–22], and among them the diketo acid (DKA, Fig. 1) class of compounds [23–25] showed the most promising derivatives. DKA-based MK-0518 (Raltegravir) and the 4-quinolone-3-carboxylic

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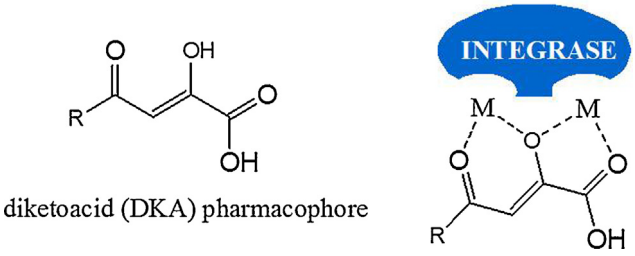

Fig. 1. DKA pharmacophore (left) and the scheme of its mode of action (right).

acid GS-9137 (Elviltegravir) (Fig. 2) [26–30] have been allowed by FDA for clinical use, confirming IN as a rational retroviral drug target.

Although several synthetic and biological studies have been reported, the mechanism of action of IN inhibitors has not been well understood. It is clear, however, that the inhibitor chelates one or, more probably, two metal ions (almost certainly Mg(II)) that are in the active site of the enzyme (Fig. 1). For this reason, we are investigating the metal complexation as a new strategy in the IN drug design and we have found that not only the ligands, but also their metal complexes show antiviral properties, modulated by the metal [7–10]. In order to gain a deeper insight on the mechanism of action of the new potential drugs, we have carefully defined the speciation in methanol/water = 9/1 solution, were the greatest part of our ligands is soluble, and measured the formation constants of their complexes with divalent metal ions of biological interest, particularly Mg(II). New elements useful to understand the mechanism of action of the ligands and their complexes could be obtained by studying the complete thermodynamics of complexes formation in solution [31–33]. Isothermal titration calorimetry (ITC) in methanol/water = 9/1 solution could provide useful information in this direction. In doing so in mixed solvent, we encountered a lot of troubles. We think it is worth noting to share our experience with the scientific community also because the literature about this subject is very limited. With this aim, herein we report the thermodynamic study of the complexes formed by N-(4-fluorobenzyl)-5-hydroxy-2-isopropyl-1-methyl-6-oxo-1,6-dihydroxypyrimidine-4-carboxylate (HL, a simplified model of Raltegravir, Fig. 2) with Mg(II), Mn(II), Co(II) and Zn(II).

2. Experimental

2.1. Materials

(HL, *N*-(4-fluorobenzyl)-5-hydroxy-1-methyl-2-(1-methyl-1-{[(5-methyl-1,3,4-oxadiazol-2-yl)carbonyl]amino}ethyl)-6-oxo-1,6-dihydropyrimidine-4-carboxamide), shown in Fig. 2,was synthesized and purified by us according to the procedure reported in Ref. [7].

The Mg(II), Mn(II), and Co(II) stock solutions were prepared from $MgCl_2 \cdot 6H_2O$ (Aldrich), $MnCl_2 \cdot 4H_2O$ (Janssen), and anhydrous $CoCl_2$ (Aldrich). Zn(II) stock solution was prepared by dissolving zinc granules (Aldrich) with HCl. Their concentrations were determined using EDTA as a titrant. For Mn(II) and Mg(II), the sodium salt of Eriochrome black T in the presence of triethanolamine and hydroxylamine chloride was used as an indicator, while for Co(II) and Zn(II), titrations were performed in the presence of sodium acetate and hexamethylenetetramine, using xylenol orange as indicator. The Co(II) solutions were prepared and stored under nitrogen to avoid oxidation. Appropriate aliquots of ligand solution were prepared by weight, using freshly boiled methanol and double-distilled water, kept under N_2.

KOH solutions in methanol/water = 9/1 at 0.1 M KCl ionic strength (0.065–0.145 M) were standardized potentiometrically against potassium hydrogen phthalate by a fully automated apparatus equipped with a CRISON GLP 21–22 digital voltmeter (resolution, 0.1 mV) and a 5 mL Metrohm Dosimat 655 autoburet and the data were processed by the Gran's method [34,35].

2.2. Isothermal titration calorimetry (ITC)

ITC measurements were carried out on a CSC model 5300 N-ITC III isothermal titration calorimeter (Calorimetry Sciences Corporations, USA) at 25 °C. KOH solution in methanol/water = 9/1 at 0.1 M

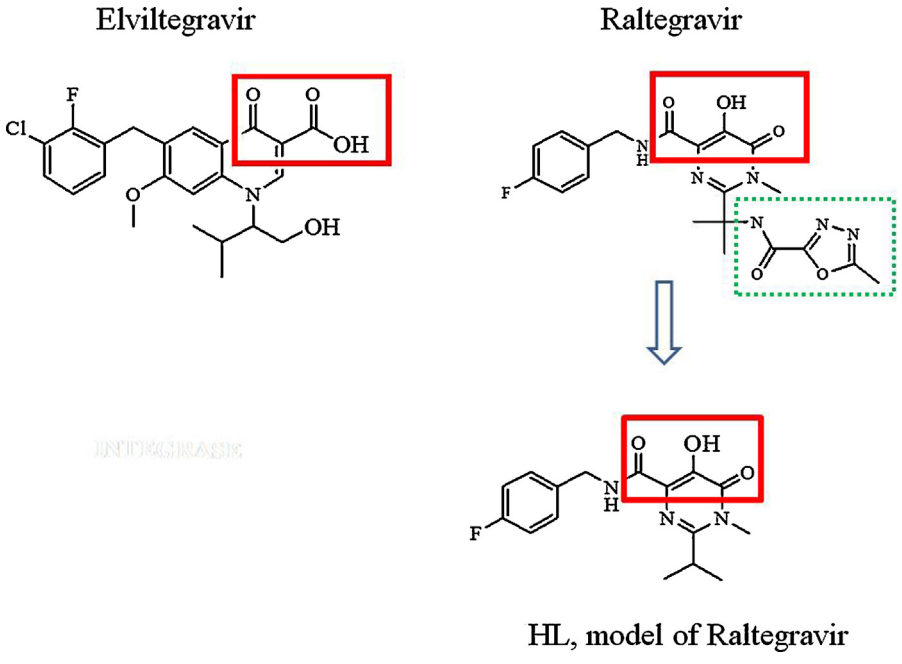

Fig. 2. Schemes of the antiretroviral drugs Elvitegravir and Raltegravir, and of the model ligand N-(4-fluorobenzyl)-5-hydroxy-2-isopropyl-1-methyl-6-oxo-1,6-dihydroxypyrimidine-4-carboxylate, HL.