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Covalent and non-covalent binding of platinated vitamin B_{12}-derivatives to a B_{12} responsive riboswitch

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With our very best wishes to Imre Sóvágó

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ABSTRACT

The B_{12} responding *btuB* riboswitch is a short, non-coding RNA sequence involved in the gene-regulation of an outer membrane B_{12}-transport protein in *E. coli*. This RNA is characterized by its selective high-affinity binding to coenzyme B_{12} and by the structural rearrangement it undergoes upon this interaction. Due to their involvement in (mostly) bacterial gene regulation, the *btuB* riboswitch as well as the further about twenty known classes of riboswitches received much attention in recent years. In case of the *btuB* riboswitch, the light sensitivity of its ligand, coenzyme B_{12}, poses one of the greater challenges for its investigation. Vitamin B_{12} derivatives carrying a cyanide-bridged platinum(II) moiety offer an ideal strategy for the design of light stable coenzyme B_{12} analogs. Developed by Alberto and coworkers in 2005 these conjugates provide the possibility to coordinate an additional nucleobase making them potential coenzyme B_{12} mimics. However, although these derivatives show a high structural similarity to coenzyme B_{12}, their functionality might differ and offers the opportunity to develop new classes of antibiotic agents. Especially the platinum(II)-complex could interact with RNA in an unexpected way, which is the main question addressed in the work presented here. We characterized the binding of three vitamin B_{12}-platinum(II) complexes to two different RNAs: the B_{12}-specific *btuB* riboswitch and the short RNA D1-45, which is a non-B_{12}-binder. cisPt(II)VitB_{12}^{+} (**1**) and enPt(II)VitB_{12}^{+} (**2**) carry both a labile chloride ligand at the platinum(II) moiety whereas dienPt(II)VitB_{12}^{2+} (**3**), that carries no labile chloride ligand anymore, can be considered chemically inert under the applied conditions. Complexes **1** and **2** covalently bind to both RNAs as monitored by band-shift assays. In the case of the short D1-45 the shifted bands are well separated and were further analyzed by MALDI-MS proving the covalent interaction. Complex **3** is unable to covalently bind any of the two RNAs, which proves the general stability of the Pt(II) complex. However, the presence of this moiety has a dramatic influence on the binding property towards the B_{12}-sensing riboswitch, as was observed in in-line probing assays by comparison to the natural ligand coenzyme B_{12}.

1. Introduction

First described in 2002 [1–4], riboswitches are just one fascinating example of relatively short but highly structured RNAs with crucial cellular function, i.e. the regulation of gene expression. Riboswitches bind with high specificity to a wide range of ligands such as metal ions, anions, aminoacids, nucleobases, or various enzymatic cofactors including one of the most complex cellular metabolites, adenosyl cobalamin (AdoCbl). This fact renders riboswitches very interesting also in a coordination chemical point of view. Hence, in the past decade these regular RNAs have drawn the attention of RNA biologist, biochemists as well as chemists.

The B_{12}-binding *btuB* riboswitch of *E. coli* was one of the first riboswitches described [2] but it took 10 years until the first crystal structures of three representatives of this riboswitch class were solved [5,6]. In general, the inherent light-sensitivity of coenzyme B_{12} represents a major challenge for any investigation of this complex system. Adapting a method developed by Alberto and coworkers [7] we earlier presented a strategy to synthesize a light-stable coenzyme B_{12} analog [8]. Vitamin B_{12} is thereby coordinated *via* its cyano ligand to a Pt(II)-complex carrying a 9-methyladenine. Replacement of the chloride ligand with other bases opens the possibility to synthesize light stable cofactor B_{12} mimics with variable chemical properties.

However, the general stability of these vitamin B_{12}-platinum(II) complexes, especially with regard of the CN-Pt and Pt-Cl bonds has not been studied. Moreover, their mode of interaction with RNA, which might possibly divert from the natural B_{12} derivatives is also

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still unknown. We therefore applied three conjugates to two different RNAs to address these questions. cisPt(II)VitB$_{12}^{+}$ (**1**) and enPt(II)VitB$_{12}^{+}$ (**2**) were synthesized as described earlier [7]. In addition, we included dienPt(II)VitB$_{12}^{2+}$ (**3**) (Scheme 1). In contrast to the derivatives **1** and **2**, the new compound **3** has no labile ligand and is expected to be chemically inert. We applied two different RNAs, first the so-called D1-45 (Fig. 1A) which has no known affinity towards B_{12}, and second the B_{12}-sensitive *btuB* riboswitch (*vide infra*). We here characterize the stability of these conjugates under typical RNA *in vitro* conditions and investigate how they interact with RNA in comparison to both, the native AdoCbl and Cisplatin itself. Furthermore, we were interested to see if these platinated VitB$_{12}$-complexes are still able to bind and switch the *btuB* riboswitch.

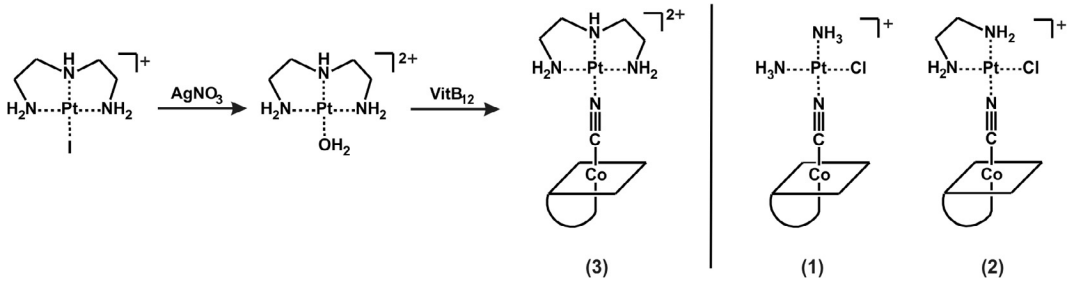
2. Experimental section

2.1. Starting materials and general procedures

cis-$(NH_3)_2PtCl_2$ (Cisplatin) [9,10] and [dienPt(II)I]I [11], were synthesized according to the literature. cisPt(II)VitB$_{12}^{+}$ (**1**) [7] and enPt(II)VitB$_{12}^{+}$ (**2**) [8] were synthesized as described, but additionally desalted on Sep-Pak® C18 cartridges from Waters (Milford MA, USA) after purification. Dichlorido(ethylenediamine)platinum (II) (en-Platin) was purchased from Alfa Aesar (Karlsruhe, Germany). Vitamin B_{12} and Coenzyme B_{12} were used without further purification as obtained from Calbiochem (San Diego CA, USA) or Sigma-Aldrich (Buchs, Switzerland) respectively. All other chemicals were of at least reagent grade purity and were used without further purification as obtained from Sigma Aldrich. The template DNA dD1-45 was bought PAGE purified from Microsynth AG (Balgach, Switzerland). The 45nt long RNA D1-45 was produced by *in vitro* transcription with homemade T7-polymerase, which was prepared and purified by a protocol adapted from literature [12]. The 202 nt long *btuB* RNA was produced by *in vitro* transcription from a plasmid [13]. Nucleoside 5′-triphosphates (ATP, GTP, CTP) and UTP were purchased from GE Healthcare (Freiburg i. Br., Germany) and Sigma-Aldrich respectively. RNase T1 1000 U/μL was purchased from Fermentas, Thermo Fisher Scientific (Waltham MA, USA) and was diluted to 1 U/μL in 50% (v/v) glycerol and 50 mM Tris-HCl (pH 7.4). The *btuB* RNA was radioactively labeled at the 5′-end with γ-^{32}P-ATP (150 mCi/mL, 6000 Ci/mmol) from PerkinElmer (Waltham MA, USA). Denaturing polyacrylamide gels were prepared using Long Ranger™ gel solution from Lonza (Rockland ME, USA). In-line probing experiments were performed as previously described [13]. All buffers, salt solutions and gel solutions used for the RNA binding assays were autoclaved or filtered through 0.2 μm filters before use.

2.2. Instrumentation

The HPLC-solvents used were 0.1% trifluoro acetic acid in H_2O (A) and methanol (B). Analytical HPLC was performed on a Hitachi Elite LaChrom system (Tokyo, Japan) equipped with a L-2400 UV detector and a Nucleodur C18 Gravity RP column (5 μm particle size) from Macherey-Nagel (Düren, Germany). The gradient used for analytical HPLC was 75% A for 5 min, linear to 29% A in 15 min, and 75% A for 5 min at 0.5 mL min^{-1} flow rate. The preparative HPLC separation was performed on a Prostar system from Varian (Palo Alto CA, USA) equipped with two Prostar 215 pumps, a Prostar 320 UV/Vis detector and a Nucleosil C-18ec RP column (7 μm particle size) from Macherey-Nagel. The gradient for the preparative HPLC was linear from 100% A to 80% A in 5 min, linear to 35% A in 40 min and then linear to 100% B in 5 min with a flow rate of 40 mL min^{-1}. IR spectra were recorded on a Perkin-Elmer Spectrum BX spectrometer in KBr pellets. ESI-MS spectra of the compounds were recorded on a Daltronics HCT instrument from Bruker (Billerica MA, USA) equipped with an Acquity Ultra Perfor-



Scheme 1. Formation of dienPt(II)VitB$_{12}^{2+}$ (**3**) by reaction of vitamin B_{12} with the activated Pt(II) complex. On the right side, schemes of cisPt(II)VitB$_{12}^{+}$ (**1**) and enPt(II)VitB$_{12}^{+}$ (**2**) are shown.

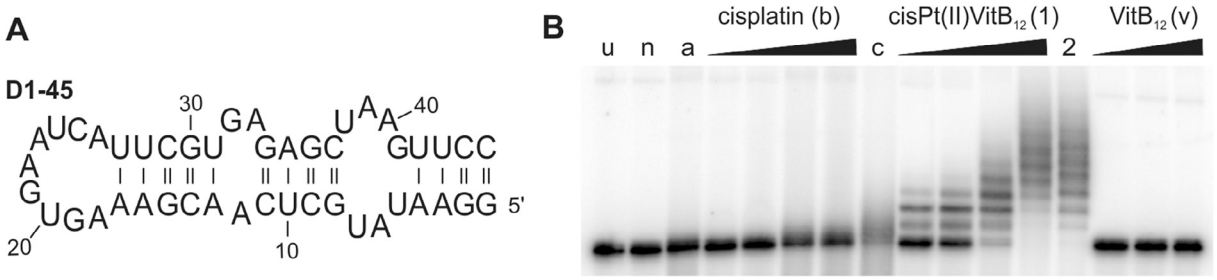


Fig. 1. Binding experiment with the B_{12}-unspecific D1-45 RNA. (**A**) The secondary structure of D1-45 which was used as a general RNA model. (**B**) Band-shift assay on denaturing PAGE depicting the influence of the different complexes on D1-45. Since the presence of up to 0.5 mM vitamin B_{12} has no influence on the RNA, a possible interaction with the conjugates is due to covalent interaction *via* the platin(II) complex. The RNA was incubated for 17h at 25°C in 50 mM Tris-HCl (pH 8.3), 20 mM $MgCl_2$ and 100 mM KCl in the absence (**n**) or in the presence of (**a**) 3 mM K_2PtCl_4, (**b**) cisplatin (0.25 to 3 mM), (**c**) 1.5 mM Pt(en)Cl_2, (**v**) Vitamin B_{12} (10 to 500 μm), (**1**) cisPt(II)VitB$_{12}^{+}$ (0.25 mM to 10 mM), or (**2**) 10 mM enPt(II)VitB$_{12}^{+}$. (**u**) is unreacted RNA.

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