CHEMBIOCHEM

DOI: 10.1002/cbic.200800099

The Corrin Moiety of Coenzyme B_{12} is the Determinant for Switching the *btuB* Riboswitch of *E. coli*

Sofia Gallo,[a] Michael Oberhuber,[b] Roland K. O. Sigel,*[a] and Bernhard Kräutler*[b]

Riboswitches are regulatory elements in the 5′-untranslated region (5′-UTR) of bacterial mRNAs that bind certain metabolites with high specificity and affinity. The 202 nucleotide (nt)-long btuB *riboswitch RNA of* E. coli *interacts specifically with coenzyme B_{12} and its derivatives thereby leading to changes in the RNA structure and hence to an altered expression of the downstream* btuB *gene. We report the investigations of the rearrangement of the three-dimensional structure of the* btuB *riboswitch upon binding to four different B_{12} derivatives: coenzyme B_{12}, vitamin B_{12}, adenosyl factor A and adenosyl-cobinamide. In-line probing experiments have shown that the corrin ring plays the crucial role in switching the three-dimensional riboswitch structure. Instead, the apical ligands influence only the binding affinity of the B_{12} derivative to the* btuB *riboswitch.*

Introduction

In the regulatory machinery of gene expression, proteins are known to function as indispensable components, as basic control elements and as sensors.[1] Recently, an alternative gene regulatory path was discovered, based on direct interactions between metabolites and specific regulatory RNAs, called "riboswitches".[2–6] These short sequences in the 5′-untranslated region (5′-UTR) of messenger RNAs (mRNAs) undergo a conformational change upon the specific binding of metabolites. Metabolite-induced restructuring of a "riboswitch" RNA leads to premature termination of transcription and/or inhibition of translation of the downstream genes, which have a close physiological relationship with the bound metabolite.[3,4]

The B_{12}-responding *btuB* riboswitch of *E. coli* is situated in the 5′-UTR of the *btuB* gene (Figure 1), which encodes an outer membrane protein that is used for the transport of B_{12} derivatives.[2,7] Coenzyme B_{12} (AdoCbl) has been suggested to be its natural metabolite-type ligand.[2] Binding of the riboswitch to the ribosome and thus expression of the *btuB* protein is repressed by AdoCbl, but not, apparently, by vitamin B_{12}.[8]

We have become interested in defining the structural basis for the interaction between coenzyme B_{12} (AdoCbl) and the *btuB* riboswitch. Here, we report on studies with AdoCbl and three related B_{12} derivatives, vitamin B_{12} ($VitB_{12}$), adenosyl-cobinamide (AdoCobi)[9] and adenosyl factor A (AdoFactA).[10] The latter three lack either the "upper" organometallic moiety or the "lower" nucleotide group of the base-coordinated ("base-on") AdoCbl, or are inherently "base-off", respectively (Scheme 1). Using AdoCobi we also wanted to test for possible coordination of a *btuB* RNA nucleobase to the Co^{III} centre of AdoCbl (or one of its analogues), similar to the coordination of a histidine imidazole observed in some B_{12}-dependent enzymes.[11–14]

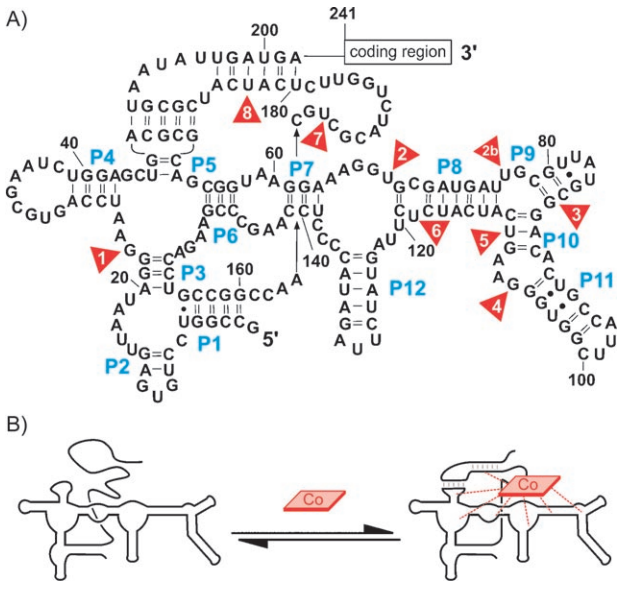
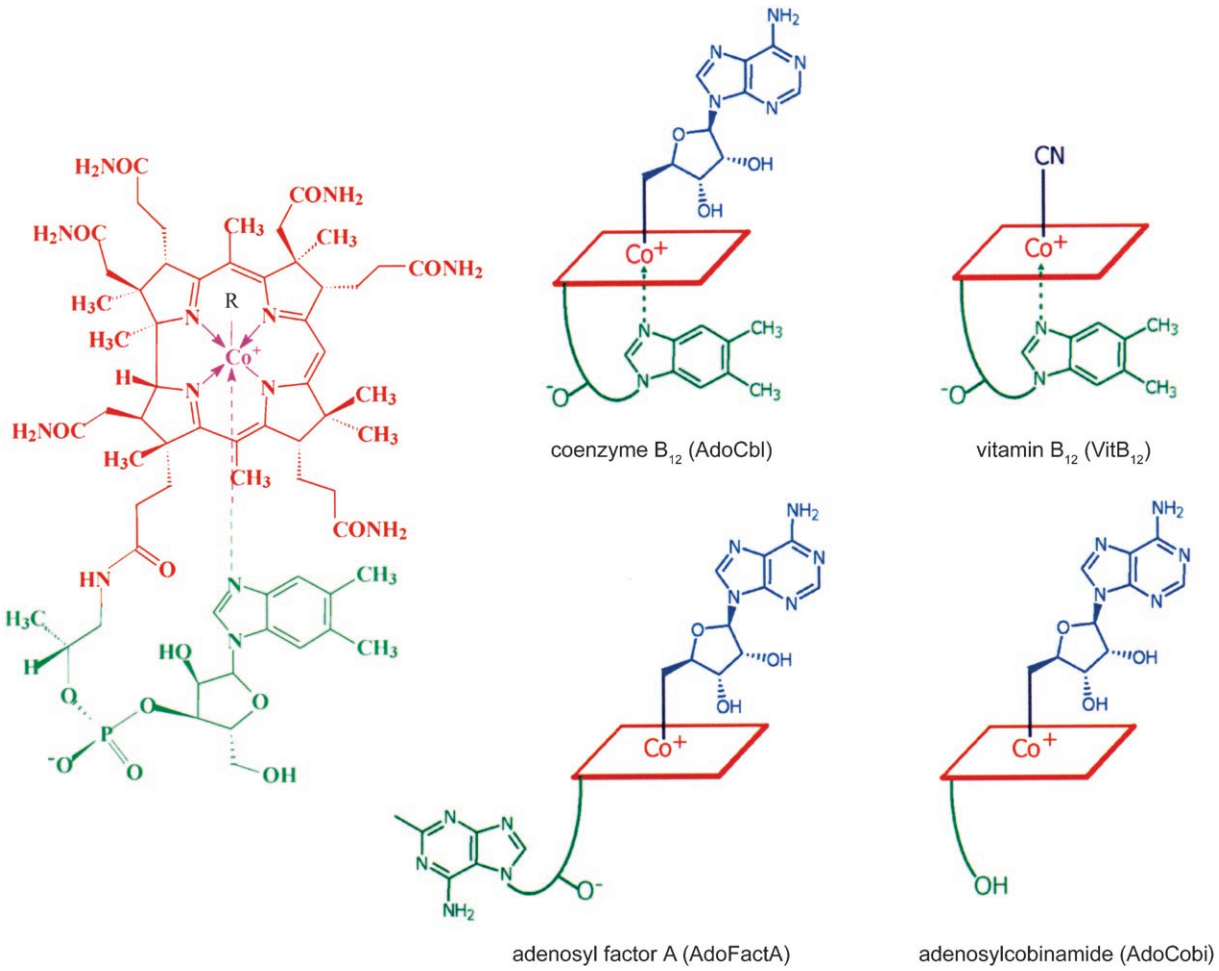


Figure 1. A) "Full-length" *btuB* B_{12} riboswitch sequence from *E. coli* as used in this study. The eight original cleavage sites of the "in-line probing" experiments are indicated by red triangles (adapted from ref. [17]) together with the newly evaluated site 2b at U77. B) A conformational change of the riboswitch is induced by binding of a suitably structured B_{12} derivative.[17,25] As shown here, the corrin moiety of AdoCbl harbours the crucial determinants; the apical groups only affect the strength of the interaction.

[a] *S. Gallo, Prof. Dr. R. K. O. Sigel*
Institute of Inorganic Chemistry, University of Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)
Fax: (+41) 44-635-68-02
E-mail: roland.sigel@aci.uzh.ch

[b] *Dr. M. Oberhuber, Prof. Dr. B. Kräutler*
Institute of Organic Chemistry and Centre of Molecular Biosciences
University of Innsbruck
6020 Innsbruck (Austria)
Fax: (+43) 512-507-2892
E-mail: bernhard.kraeutler@uibk.ac.at



Scheme 1. B_{12} derivatives used in this study: structural formulae of coenzyme B_{12} (AdoCbl, R = 5′-deoxyadenosyl) and vitamin B_{12} ($VitB_{12}$, R = CN), and symbolic structures of "base-on" AdoCbl and $VitB_{12}$, "base-off" adenosyl-factor A (AdoFactA) and "incomplete" adenosyl-cobinamide (AdoCobi).

Results and Discussion

The deduced structural changes of the *btuB* riboswitch are due to the corrin moiety of bound B_{12} derivatives

"In-line probing"—a method to map conformational changes in RNA structures[2,15,16]—has been used to monitor the binding of AdoCbl to the "full-length" *btuB* riboswitch, which comprises the first 202 nucleotides (nt) of the *btuB* 5′-UTR. Binding of AdoCbl led to a conformational switch of the *btuB* RNA. This switch was reflected by the appearance or disappearance of eight distinct cleavage bands in a manner dependent upon the metabolite concentration. These bands were distributed over the whole *btuB* riboswitch sequence; this emphasizes the relevance of the "full-length" riboswitch for high-affinity recognition of this bound B_{12} cofactor.[2] Binding of AdoCbl occurred rather specifically and with high affinity.[2,8,17] However, no interaction with *btuB* RNA was detected for $VitB_{12}$, methylcobalamin and dicyano-cobinamide.[2,17]

To test for the structural requirements of the metabolite to switch the riboswitch, we investigated the binding of AdoCbl and of the B_{12} derivatives, $VitB_{12}$, AdoFactA and AdoCobi (Scheme 1) to *btuB* RNA (202 nt) over a wide concentration range of the corrinoid by using "in-line probing" experiments. After incubation of the [^{32}P]-5′-end-labelled *btuB* RNA with each of the four B_{12} derivatives, the cleavage products were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). Our studies with AdoCbl reproduced the earlier observations qualitatively.[2] As reported earlier,[2,17] the intensities of the bands 1, 3, 4 and 8 decreased upon addition of AdoCbl, whereas the intensities of the bands 2, 5, 6 and 7 increased; this indicates accelerated strand cleavage at these latter sites (Figures 2 and 3). In addition, we found one further cleavage band at U77, which slightly decreased its intensity (band 2b in Figures 1, 2 and 3).

However, when applying the other three B_{12} derivatives in such "in-line probing" experiments, all of them were found to display the same RNA cleavage pattern as was observed for AdoCbl,[2] including the new band 2b, although higher concentrations were needed in the case of AdoCobi and $VitB_{12}$ (Figures 2 and 3). AdoFactA is a naturally occurring analogue of AdoCbl in which dimethylbenzimidazole (DMB) is replaced by 2-methyladenine as the nucleotide base.[10,18] In contrast to AdoCbl and $VitB_{12}$, which both display the "base-on" form in neutral aqueous solution, AdoFactA preferentially exists in the

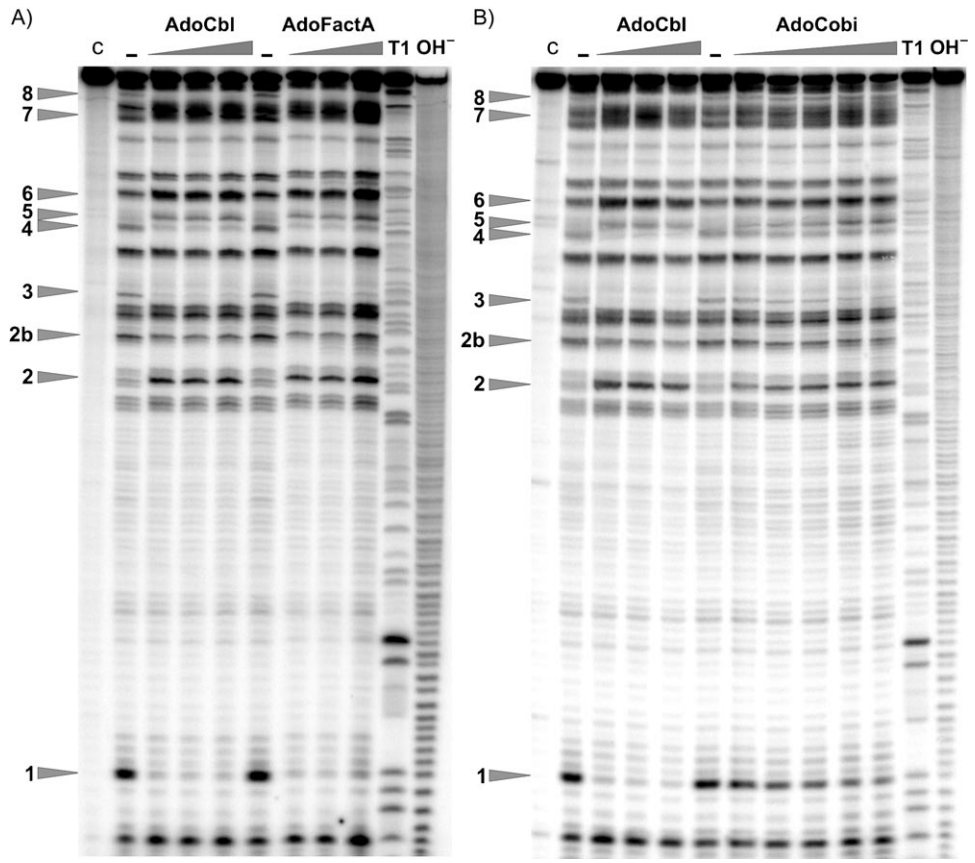


Figure 2. In-line probing experiments of the 202-nt-long *btuB* riboswitch with A) AdoFactA and B) AdoCobi by using AdoCbl as reference. Lane c : control; T1: RNAse T1 digestion; OH^-: alkaline hydrolysis ladder. The *btuB* riboswitch RNA was incubated for 40 h in the absence (–) or the presence of 0.1, 0.5 and 1 mM AdoCbl or AdoFactA, or with 0.5, 1, 2, 5 and 8 mM AdoCobi (see also the Experimental Section). The arrows indicate the bands that changed their intensities upon addition of B_{12}:[2,17] G23 (1), U68 (2), U77 (2b), G87 (3), G106 (4), U110 (5), U118 (6), U167 (7), U183 (8).

"base-off" form.[10] Interestingly, AdoFactA was found here to bind readily to the *btuB* RNA and also to induce the same conformational switch (Figure 2A, Table 1). AdoCobi, which lacks the entire nucleotide portion of AdoCbl, also induces essentially the same cleavage pattern of *btuB* RNA, as AdoCbl and $VitB_{12}$ (the fourth B_{12} derivative tested here). However, the change in the cleavage intensity of bands 5, 7 and 8 are different compared to the other three derivatives that were used (Figures 2B and 4C); this suggests a possible interaction of these regions within the riboswitch with the "lower" parts of B_{12} derivatives.

All four B_{12} derivatives were thus found to induce essentially the same conformational changes in the structure of the *btuB* riboswitch, as reflected by the change of intensities of nine bands upon addition of the B_{12} derivatives (Figures 2 and 3). Aside from the concentration-dependent changes of these nine distinct bands, no further differences in cleavage bands within the 202 nucleotides were observed with either B_{12} derivative. These findings indicate that the corrin moiety of AdoCbl is responsible for the structural changes within the *btuB* riboswitch rather than the large functional upper or lower axial ligands that are bound at the cobalt centre.

The organometallic adenosine moiety and the nucleotide loop enhance the affinity of AdoCbl for the *btuB* riboswitch

To explore the possible effect of the organometallic adenosine moiety and of the nucleotide loop of AdoCbl on its binding affinity towards the *btuB* riboswitch, ^{32}P-labelled RNA was incubated with the four B_{12} derivatives (Figures 2 and 3) and the band-intensities were registered as a function of the concentration of the respective corrinoid. The resulting plots could be fitted to a 1:1 binding isotherm model[19] (Figure 4), which yielded the affinities for each B_{12} derivative: $\log K_{av,AdoCbl} = 7.05$, $\log K_{av,VitB12} = 3.50$, $\log K_{av,AdoFactA} = 6.54$, $\log K_{av,AdoCobi} = 3.12$ (Table 1). In the earlier work with *btuB* RNA,[2] similar affinities were determined for AdoCbl ($K_D \approx$ 300 nM), whereas evidence for binding of $VitB_{12}$ (at lower concentration) to this B_{12} riboswitch was not found.[2,8,17] AdoCbl and AdoFactA bind to the B_{12} riboswitch from *E. coli* with roughly similar affinities (Table 1). As deduced for all nine observed cleavage sites along the *btuB* RNA sequence, the induced concentration-dependent conformational change due to AdoCbl or AdoFactA is the same. This implies a correlated conformational rearrangement of the RNA upon binding to each of the two "complete" Ado-corrinoids. Apparently, the "base-off" nature of AdoFactA has little influence on the binding and the reorganization of *btuB* RNA. On the other hand, $VitB_{12}$ and AdoCobi, which lack either the upper organometallic group or the lower base, both bind about 1000 times weaker than AdoCbl and AdoFactA. The derived binding affinities of $VitB_{12}$ and AdoCobi imply that the axial groups at the cobalt centre in AdoCbl are important to reach a highly stable complex with the RNA. Along this line, dicyano-cobinamide, which lacks both large axial ligands does not bind to the *btuB* RNA up to a concentration of 16 mM (data not shown). In the cases of $VitB_{12}$ and AdoCobi, the affinities obtained from the eight bands are similar, except for those from band 1 of $VitB_{12}$ and band 5 of AdoCobi (Table 1). As suggested by the different intensities of some of these cleavage bands, these regions of the riboswitch might be in contact with the "upper" and "lower" parts of AdoCbl, which are not present in either $VitB_{12}$ or AdoCobi, respectively. Alternatively, an indirect influence is exhibited as a result of the binding of one of the latter B_{12} derivative.

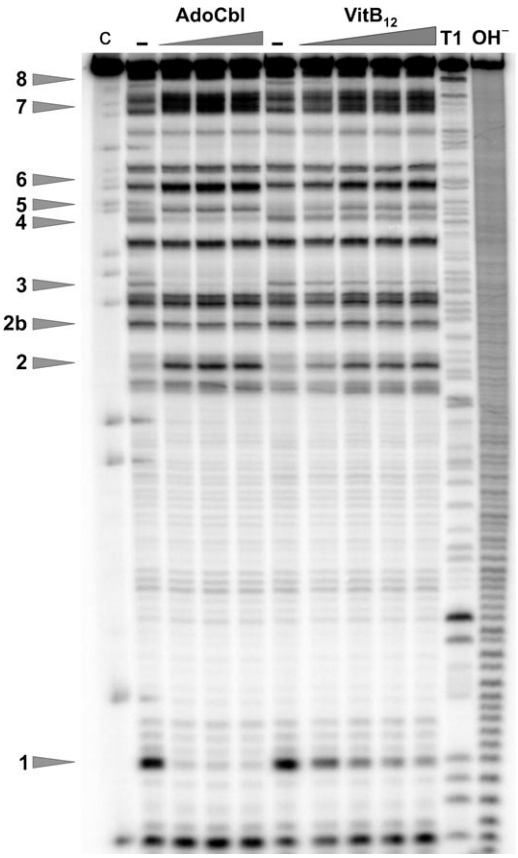


Figure 3. "In-line probing" experiment of the 202-nt-long *btuB* riboswitch. The denaturing polyacrylamide gel of the 5′ end ^{32}P-labelled riboswitch is shown after incubation for 41 h with increasing amounts of either AdoCbl (lanes 2 to 5) or $VitB_{12}$ (lanes 6 to 10). Lane c: control, that is, the riboswitch in the absence of monovalent and divalent metal ions as well as B_{12} derivative; T1: RNAse T1 digestion; OH^-: alkaline hydrolysis ladder. The concentrations of AdoCbl was zero (−), 0.1, 0.5 and 1 mM, and of $VitB_{12}$ zero (−), 0.1, 0.5, 1 and 10 mM. The cleavage bands that changed their intensities upon addition of B_{12} are indicated and are located at positions G23 (1), U68 (2), U77 (2b), G87 (3), G106 (4), U110 (5), U118 (6), U167 (7), U183 (8).

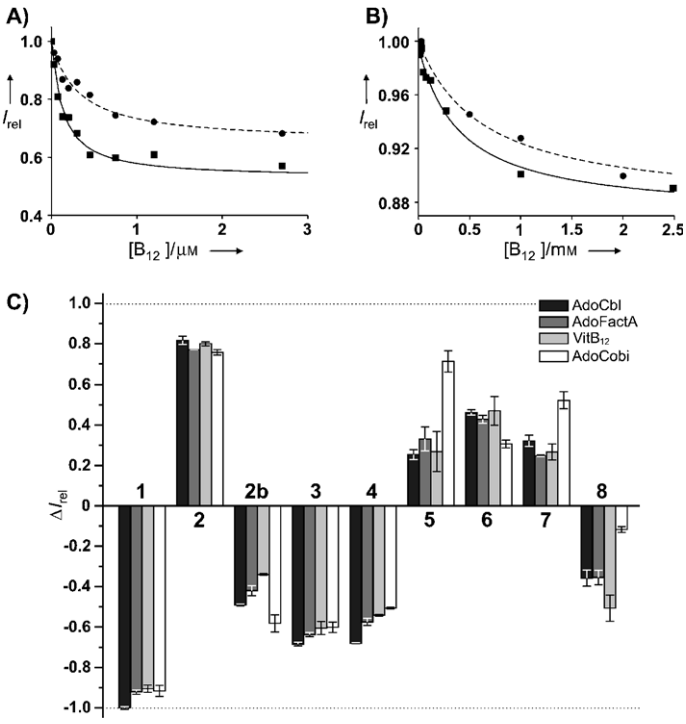


Figure 4. Cleavage pattern of full-length *btuB* RNA in "in-line probing" experiments. Change in intensity of cleavage band 3 (G87) of the *btuB* riboswitch with respect to increasing amounts of a B_{12} derivative as observed: A) AdoCbl (■) and AdoFactA (●) exhibit a much higher affinity towards the RNA than B) $VitB_{12}$ (■) and AdoCobi (●). The experimental data points were fitted to a 1:1 binding isotherm model.[19] C) Relative maximal changes in cleavage intensity of the nine bands evaluated upon addition of either of the four B_{12} derivatives AdoCbl, AdoFactA, $VitB_{12}$ and AdoCobi. The intensities shown correspond to the calculated intensities of the fully bound species, as obtained by a 1:1 binding isotherm model[19] (see also the Experimental Section), and are shown relative to the maximally observed change of band 1 upon addition of AdoCbl to the *btuB* riboswitch. The values depicted correspond to the weighted mean of the results that were obtained from two experiments with $VitB_{12}$, three experiments each with AdoCbl and AdoFactA, and six experiments with AdoCobi. In the case of band 8 of AdoCobi, the results from only one experiment could be used for the fit, but the decrease in intensity was generally observed. The error limits shown correspond to one standard deviation.

Table 1. The affinities $\log K$ of the four B_{12} derivatives to the full-length *btuB* riboswitch. The values derived from each individual cleavage site are listed (Figure 2), together with the affinities $\log K_{av}$ (and corresponding K_D) of each derivative to the riboswitch. The individual values for each band are the weighted mean of several independent experiments; all errors correspond to one standard deviation (see also the Experimental Section and legend to Figure 4).

Site	$\log K_{AdoCbl}$	$\log K_{AdoFactA}$	$\log K_{VitB12}$	$\log K_{AdoCobi}$
1	7.11 ± 0.05	6.63 ± 0.02	4.09 ± 0.02	3.14 ± 0.02
2	7.06 ± 0.05	6.37 ± 0.09	3.39 ± 0.22	3.41 ± 0.04
2b	7.13 ± 0.10	6.60 ± 0.04	3.52 ± 0.29	2.78 ± 0.14
3	7.04 ± 0.05	6.61 ± 0.04	3.45 ± 0.08	3.27 ± 0.04
4	7.06 ± 0.03	6.44 ± 0.11	3.47 ± 0.18	3.31 ± 0.01
5	6.87 ± 0.40	6.58 ± 0.21	3.21 ± 0.48	2.46 ± 0.16
6	7.01 ± 0.03	6.62 ± 0.09	3.37 ± 0.31	3.52 ± 0.06
7	7.00 ± 0.07	6.72 ± 0.20	3.40 ± 0.50	3.14 ± 0.64
8	6.52 ± 1.63	6.27 ± 0.07	3.63 ± 0.11	3.08 ± 0.12
$\log K_{av}$	7.05 ± 0.03	6.54 ± 0.05	3.50 ± 0.20	3.12 ± 0.11
K_D	89 ± 6 nM	290 ± 32 nM	314 ± 141 μM	753 ± 189 μM

The *btuB* RNA does not provide a cobalt-coordinating ligand for AdoFactA and AdoCobi

The experiments described here and elsewhere[2,17] suggested the relevance of the nucleotide loop of AdoCbl for tight binding to the *btuB* RNA. Whereas AdoCbl, AdoFactA, $VitB_{12}$ and AdoCobi all were found here to induce essentially the same structural changes in this riboswitch, a still unanswered question concerns the structure of the bound B_{12} derivatives themselves. Indeed, some of the enzymes that are dependent on AdoCbl bind the corrinoid "base-off/his-on", that is, the DMB ligand of coenzyme B_{12} is replaced by the imidazole of a histidine residue.[11,20,21] Likewise, all known methyl-transferases bind the methyl-corrinoid cofactors "base-off".[12,13] Hence, we were particularly interested in the question of how AdoCbl and AdoFactA ("molecular switches")[22] are bound to the *btuB* riboswitch.

At neutral pH and at room temperature coenzyme B_{12} (AdoCbl) and vitamin B_{12} ($VitB_{12}$) exist nearly exclusively in a "base-on" constitution. In contrast, AdoFactA prevails to about 70% in the "base-off" form, in which the "lower" adenine ligand is de-coordinated.[10] The isomeric forms can be distinguished readily by UV/visible spectroscopy: in aqueous solution, "base-on" AdoCbl displays a maximal absorbance near 525 nm, whereas the UV/visible spectrum of AdoFactA shows an absorbance maximum near 460 nm, due to its dominant "base-off" constitution (see for example, ref. [23] and Figure 5). AdoFactA has been used before as a probe for the possible restructuring of the lower ligand of B_{12} by its environment.[23,24]

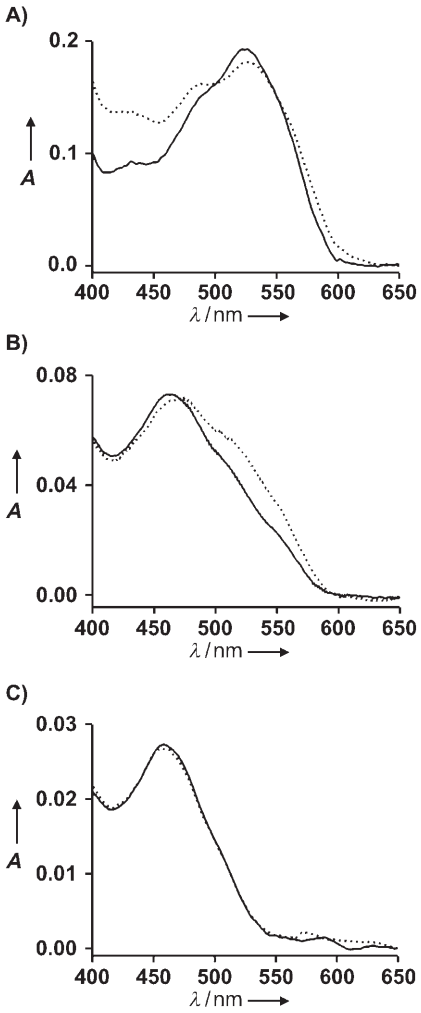


Figure 5. Comparison of the UV spectra of AdoCbl, AdoFactA and AdoCobi alone (——) and in the presence (····) of the 202-nt-long *btuB* riboswitch from *E. coli*. A) Spectra of AdoCbl (25.3 μM) in the presence (75.3 μM; that is 99.8% AdoCbl bound) or absence of *btuB*-RNA, both show an absorbance maxima at 524 nm; B) spectra of AdoFactA (7.7 μM) in the presence (30.8 μM; that is, 98.6% AdoFactA bound) or absence of *btuB* RNA show absorbance maxima at 463 and 470 nm, respectively; C) spectra of AdoCobi (1 μM) in the presence (302.4 μM; that is, 30% AdoCobi bound) or absence of *btuB* RNA, both show absorbance maxima at 457 nm. The slight (relative) absorbance changes between 500 and 550 nm might be due to changes in the coordinative environment of the corrin-bound cobalt centre (see for example ref. [24]).

The absorbance spectra of AdoCbl undergo only minor changes in the presence of an excess of *btuB* RNA; this indicates that this B_{12} derivative remains "base-on" when bound to the B_{12} riboswitch (Figures 5 and 6). In contrast, a solution of AdoFactA and excess of *btuB* RNA (under conditions in which >98% of AdoFactA was RNA-bound; Table 1) exhibits an absorbance maximum near 460 nm. This shows that this analogue of AdoCbl is largely "base-off" when bound to the B_{12} riboswitch, and indicates that *btuB* RNA is switched by these complete corrinoids, irrespective of their "base-on" or "base-off" state. Likewise, the absorbance spectrum of 1 μM AdoCobi is hardly changed in the presence of 302 μM *btuB* RNA (that is, under conditions where 30% of the corrinoid are bound, Table 1 and Figure 5). Axial (intermolecular) coordination of an RNA-based nitrogen atom as a ligand to the corrin-bound cobalt centre of the "incomplete" corrinoid AdoCobi (or of "base-off" AdoFactA), is thus a minor contributor at most, and of insignificant structural relevance.

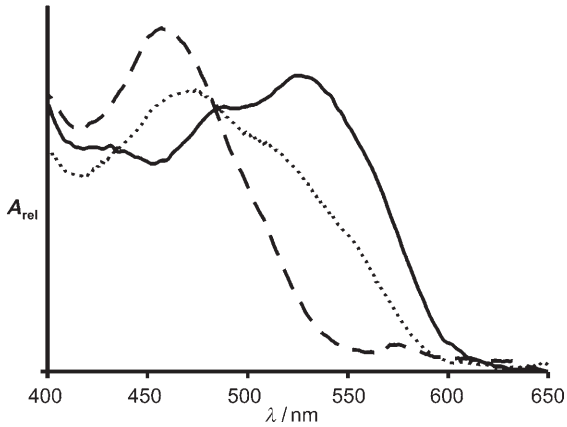


Figure 6. UV/Vis spectra of AdoCbl (——), AdoFactA (····) and AdoCobi (-----) in the presence of the *btuB* riboswitch. AdoCbl displays the "base-on" form with a maximum absorbance at 524 nm, while the maxima at 468 nm (AdoFactA) and 457 nm (AdoCobi) indicate the "base-off" forms. The B_{12}:RNA ratio was such that >98% (in case of AdoCbl and AdoFactA) or 30% (AdoCobi) of the corrinoids were bound to the riboswitch (50 mM EPPS, pH 7.5, 25 °C, 100 mM KCl, 20 mM $MgCl_2$); the intensities of the three absorption traces are not mutually normalized (see the Experimental Section for details).

Conclusions

We address here the problem of how a B_{12} riboswitch RNA[26] and coenzyme B_{12} (AdoCbl)[27] recognize and bind each other specifically. Both of these binding partners stand out as highly complex representatives of their two functional classes of biomolecules. Other metabolite-binding riboswitches have meanwhile been characterized by X-ray crystallography (e.g., the *S*-adenosyl-methionine (SAM)-binding riboswitch)[28] and were found to bind their target metabolites in a rather tight complex, which is consistent with the deduced recognition determinants of SAM-binding riboswitches.[29]

AdoCbl and the related B_{12} derivatives $VitB_{12}$, AdoFactA and AdoCobi were all shown here to bind and induce essentially the same overall conformational change of the *btuB* riboswitch of *E. coli*. Thus, the corrin moiety of AdoCbl is the main structural determinant for inducing the *btuB* RNA to switch. This contrasts the conclusions from the previous studies, which suggested that the "upper" and "lower" ligands of AdoCbl were both also crucial for binding by this B_{12} riboswitch and for inducing its conformational restructuring.[2,8] In these studies,[2] only close analogues of AdoCbl (such as 2′-deoxy-AdoCbl and purinyl-Cbl) were deduced to bind and induce the switch effectively, while $VitB_{12}$ was noted not to bind. However, both of the two "complete" Co_{β}-adenosyl-corrinoids tested here, AdoCbl and AdoFactA, bind in the (physiologically most relevant) submicromolar range. Clearly, *btuB* RNA is neither specific for the DMB base nor for the "base-on" state of AdoCbl. In addition, $VitB_{12}$ and AdoCobi, which either lack the large organometallic group ($VitB_{12}$), or are "incomplete" (AdoCobi), both induced the conformational switch of the *btuB* RNA also, but exhibited a more than 1000-fold weaker binding affinity.

In fact, neither one of the large axially bound moieties is needed to induce the conformational switch of *btuB* RNA, in spite of their strongly enhancing effect on the binding affinity. Only when both large ligands are removed—as is the case with dicyano-cobinamide—does the affinity become so small that no binding occurs under the conditions applied. Metabolite-binding riboswitches have been suggested to have a major two-domain topology, with an aptamer domain and an expression platform.[26] The 5′-UTR of the *btuB* RNA (and other B_{12} riboswitches) contain a conserved 25-nt sequence, the "B_{12} box",[30] as part of the highly conserved and longer "B_{12} element".[25] The identification of (most of) these B_{12} riboswitches by sequence comparisons also revealed a large conserved stem–loop region (called the BII-element in ref. [25]), which is not available in all B_{12} riboswitches.[17,25] Our results indicate that the aptamer domain of the *btuB* B_{12} riboswitch of *E. coli* has a modular build-up, and can apparently provide two types of binding interface to the corrinoid: one of them can be switched by the bound B_{12}, the other can recognize relevant parts of the structure of the two large apical moieties of AdoCbl and increase the binding affinity further without detectable conformational restructuring (an earlier suspected feature of the *btuB* B_{12} riboswitches[17]).

The deduced ability of natural B_{12} derivatives to switch *btuB* RNA correlates remarkably with the binding affinity of the (proteinous) outer-membrane transporter *btuB*, itself.[8,31] In view of the biosynthetic capacities of *E. coli*, which can use cobinamides for the enzyme-catalyzed build-up of the metabolically useful B_{12} cofactors (methylcobalamin and AdoCbl), the similar import of cobinamides and cobalamins via *btuB* is economical.[31] The related ability of the *btuB* B_{12} riboswitch of *E. coli* to bind a broad range of natural B_{12} derivatives and for being switched by "incomplete" corrinoids (such as AdoCobi) and natural "base-off" cobamides (as AdoFactA) thus is consistent, in a rather fascinating way, with the apparent structural tolerance in the B_{12} uptake by *E. coli*.

Experimental Section

Materials: Coenzyme B_{12} (Fluka) and vitamin B_{12} (Calbiochem) were used without further purification. Adenosyl factor A and adenosylcobinamide were prepared by an electrochemical method.[10,32] Nucleoside 5′-triphosphates were purchased from GE Healthcare, except for UTP, which was obtained from Fluka–Sigma–Aldrich. The T7 RNA polymerase that was used for in vitro transcription was prepared according to ref. [33]. Denaturing polyacrylamide gels were prepared by using long ranger gel solution (Cambrex Bio Science, Rockland, ME, USA). Deionised water was further purified by Millipore-Filtration and autoclaved before use, and all buffers, salt solutions and gels were filtered through 0.2 μm filters. All other chemicals used were at least puriss p.a., and were purchased from either Fluka–Sigma–Aldrich or Brunschwig Chemie. Gels were analyzed with a Storm860 PhosphoImager and ImageQuant software and the UV/Vis spectra were recorded either by using a Varian Cary 500 or a Hitachi U-3000 spectrophotometer.

Preparation of the RNA: The DNA that encoded the 202-nucleotide-long *btuB* riboswitch sequence was isolated directly from the bacterial genome of *E. coli* K12 by colony PCR and was subsequently cloned between a 5′-hammerhead and a 3′-hepatitis delta virus ribozyme sequence, to yield after in vitro transcription and ribozyme self cleavage the riboswitch with uniform ends.[33,34] After in vitro transcription of this pSG1 plasmid derived from the pRZ-vector[34] with T7 polymerase, the ribozymes were cut out by thermal cycling with $MgCl_2$ (40 mM).[33,34] The RNA was subsequently purified with denaturing PAGE (10%), isolated by electroelution at 4 °C, and concentrated by centrifugation (Vivaspin) as described before.[33] The RNA was stored in water at −20 °C.

"Inline-probing" experiments: "In-line probing" experiments were performed by using ^{32}P-5′-labelled RNA (~5 nM ^{32}P-labelled RNA of an overall RNA concentration of 20 nM). The RNA was incubated for 41 h in the dark at 25 °C (50 mM Tris–HCl, pH 8.3, 20 mM $MgCl_2$, 100 mM KCl) and in the presence or absence of the corresponding B_{12} derivative. The cleaved bands were separated by denaturing PAGE (10%, 7 M urea).

The titration experiments with AdoCbl and AdoFactA were performed under conditions described above by addition of 0, 30, 75, 130, 200, 300, 450, 750 nM, 1.2, 2.7, 10 and 100 μM AdoCbl or AdoFactA to an overall RNA concentration of 37.39 nM (AdoCbl) or 30.16 nM (AdoFactA). The titrations with $VitB_{12}$ were made by addition of 0, 3.3, 7.5, 12.9, 20, 30, 45, 75, 120, 270 μM, 1 and 2.5 mM $VitB_{12}$ to the RNA (30.16 nM; Figure 3). The titrations with AdoCobi were performed by addition of 0, 0.5, 1, 2, 5 and 8 mM AdoCobi to the RNA (20 nM). The concentrations applied with dicyano-cobinamide were 0.5, 1, 2, 4, 8 and 16 mM (20 nM RNA), but no binding could be detected. Three titration experiments each were done with AdoCbl, AdoFactA and dicyano-cobinamide, two with $VitB_{12}$, and six with AdoCobi.

The changes at each cleavage site were quantified by plotting the intensities of the bands after background-correction. The experimental data points were fitted to a 1:1 binding isotherm[19] to give the K_a values of the B_{12} derivatives to the different sites. The individual values of the affinity constants for each band correspond then to the weighted mean of three independent experiments (two in the case of $VitB_{12}$). The final $\log K_{av}$ values for each B_{12} derivative are either the weighted (AdoCbl) or the arithmetic mean (AdoFactA, AdoCobi, $VitB_{12}$) of the values for each band. All errors given correspond to one standard deviation.

Acknowledgements

This work was supported by the Swiss National Science Foundation (SNF-Förderungsprofessur PP002-114759/1 and project 200021-117999/1 to R.K.O.S.), by a Forschungskredit of the University of Zürich (S.G.) and by the Austrian National Science Foundation (FWF proj. No. 13595, to B.K.).

Keywords: B_{12} riboswitch · coenzyme B_{12} · gene expression · riboswitches · RNA

[1] R. Knippers, *Molekulare Genetik*, 8th ed., Thieme, Stuttgart **2001**.
[2] A. Nahvi, N. Sudarsan, M. S. Ebert, X. Zou, K. L. Brown, R. R. Breaker, *Chem. Biol.* **2002**, *9*, 1043–1049.
[3] A. G. Vitreschak, D. A. Rodionov, A. A. Mironov, M. S. Gelfand, *Trends Genet.* **2004**, *20*, 44–50.
[4] M. Mandal, R. R. Breaker, *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 451–463.
[5] W. C. Winkler, R. R. Breaker, *ChemBioChem* **2003**, *4*, 1024–1032.
[6] W. C. Winkler, A. Nahvi, A. Roth, J. A. Collins, R. R. Breaker, *Nature* **2004**, *428*, 281–286.
[7] A. Gudmundsdottir, C. Bradbeer, R. J. Kadner, *J. Biol. Chem.* **1988**, *263*, 14224–14230.
[8] X. Nou, R. J. Kadner, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7190–7195.
[9] B. P. Hay, R. G. Finke, *J. Am. Chem. Soc.* **1987**, *109*, 8012–8018.
[10] W. Fieber, B. Hoffmann, W. Schmidt, E. Stupperich, R. Konrat, B. Kräutler, *Helv. Chim. Acta* **2002**, *85*, 927–944.
[11] F. Mancia, N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bosecke, O. Diat, P. R. Evans, *Structure* **1996**, *4*, 339–350.
[12] C. L. Drennan, S. Huang, J. T. Drummond, R. G. Matthews, M. L. Ludwig, *Science* **1994**, *266*, 1669–1674.
[13] C. L. Drennan, R. G. Matthews, M. L. Ludwig, *Curr. Biol. Curr. Op. Struct. Biol.* **1994**, *4*, 919–929.
[14] K. Gruber, C. Kratky, *Curr. Opin. Chem. Biol.* **2002**, *6*, 598–603.
[15] G. A. Soukup, R. R. Breaker, *RNA* **1999**, *5*, 1308–1325.
[16] G. A. Soukup, E. C. DeRose, M. Koizumi, R. R. Breaker, *RNA* **2001**, *7*, 524–536.
[17] A. Nahvi, J. E. Barrick, R. R. Breaker, *Nucleic Acids Res.* **2004**, *32*, 143–150.
[18] H. A. Barker, A. A. Iodice, V. Rooze, F. Suzuki, *J. Biol. Chem.* **1964**, *239*, 3260–3266.
[19] R. K. O. Sigel, E. Freisinger, B. Lippert, *J. Biol. Inorg. Chem.* **2000**, *5*, 287–299.
[20] M. L. Ludwig, P. R. Evans in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, p. 595.
[21] R. Reitzer, K. Gruber, G. Jogl, U. G. Wagner, H. Bothe, W. Buckel, C. Kratky, *Structure* **1999**, *7*, 891.
[22] S. Gschösser, K. Gruber, C. Kratky, C. Eichmüller, B. Kräutler, *Angew. Chem.* **2005**, *117*, 2324–2328; *Angew. Chem. Int. Ed.* **2005**, *44*, 2284–2288.
[23] R. B. Hannak, R. Konrat, W. Schüler, B. Kräutler, M. T. M. Auditor, D. Hilvert, *Angew. Chem.* **2002**, *114*, 3765–3768; *Angew. Chem. Int. Ed.* **2002**, *41*, 3613–3616.
[24] S. N. Fedosov, N. U. Fedosova, B. Kräutler, E. Nexø, T. E. Petersen, *Biochemistry* **2007**, *46*, 6446–6458.
[25] A. G. Vitreschak, D. A. Rodionov, A. A. Mironov, M. S. Gelfand, *RNA* **2003**, *9*, 1084–1097.
[26] W. C. Winkler, R. R. Breaker, *Annu. Rev. Microbiol.* **2005**, *59*, 487–517.
[27] B. Kräutler in *Vitamin B_{12} and B_{12} Proteins* (Eds.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley, Weinheim, **1998**, p. 3.
[28] R. K. Montange, R. T. Batey, *Nature* **2006**, *441*, 1172–1175.
[29] J. Lim, W. C. Winkler, S. Nakamura, V. Scott, R. R. Breaker, *Angew. Chem.* **2006**, *118*, 978–982; *Angew. Chem. Int. Ed.* **2006**, *45*, 964–968.
[30] C. V. Franklund, R. J. Kadner, *J. Bacteriol.* **1997**, *179*, 4039–4042.
[31] C. Bradbeer in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, p. 489.
[32] B. Kräutler in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, p. 315.
[33] S. Gallo, M. Furler, R. K. O. Sigel, *Chimia* **2005**, *59*, 812–816.
[34] S. C. Walker, J. M. Avis, G. L. Conn, *Nucleic Acids Res.* **2003**, *31*, e82.

Received: February 14, 2008
Published online on May 28, 2008

