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Tetrazole derivatives and matrices as novel cobalamin coordinating compounds

Sergey N. Fedosov [a,*], Lars Berglund [b], Ebba Nexø [c], Torben E. Petersen [a]

[a] Protein Chemistry Laboratory, Department of Molecular Biology, University of Aarhus, Science Park, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark
[b] Cobento A/S, Science Park, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark
[c] Department of Clinical Biochemistry, AS, Aarhus University Hospital, Nørrebrogade 44, 8000 Aarhus C, Denmark

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Abstract

Cobalamin (Cbl, vitamin B_{12}) consists of two moieties: (i) the corrin ring with the central Co-ion in the oxidation states $Co^{3+/2+/1+}$ and (ii) the nucleotide side chain. The lower position of the ring is typically occupied by the nucleotide base (Bzm), whereas the upper surface coordinates exchangeable ligands. We have found that amino-tetrazole can coordinate to $H_2O \cdot Cbl$ (Co^{3+}) with $K_d = 10^{-5}$–10^{-6} M. A specific group (presumably tetrazole, TZ) can be easily created in CNBr-activated Sepharose by treatment with N_3^-. The prepared matrix (STZ) contained ≈10 mM of the active groups, which bound $H_2O \cdot$ corrinoids with $K_d = 10^{-5}$–10^{-6} M. Stability of STZ–Cbl bonds gradually increased and reached $K_d = 10^{-7}$ M over 10–20 h (20 °C, pH 6–7). This effect can be ascribed to partial displacement of Bzm and coordination of TZ to the lower position. The binding was most efficient at pH 4–7 and low ionic strength, yet, noticeable adsorption took place even at extreme conditions, pH 1–9 and $I = 0$–2 M. Reduced corrins (Co^{2+}) also exhibited high affinity for STZ. The bound ligands could be eluted as $H_2O \cdot Cbl$ (pH 0), $HO \cdot Cbl$ (pH 14) or $diCN \cdot Cbl$ (pH 9–12, CN^-). The adsorbent is applicable for one-step purification of corrins from a crude extract; separation of aquo- and diaquo-forms; specific capturing of $H_2O \cdot Cbl$ from a mixture containing organo-Cbls or protein-bound Cbl, analysis of peptide-Cbl dissociation kinetics, etc.

Keywords: Tetrazole; Amino-tetrazole; Azide; Cobalamin; Corrinoid; Coordination; Adsorbent; Sepharose

1. Introduction

Vitamin B_{12} (or cobalamin, Cbl) is an organometallic cofactor of complex structure [1,2]. It is synthesised only by bacteria, and all other organisms obtain the vitamin via a complicated food chain [3,4]. Insufficiency of vitamin B_{12} in humans causes severe disorders accompanied by neurological abnormalities, anaemia and death, if not treated [3,4].

The core architecture of Cbl (Fig. 1a) includes the corrin ring with a central cobalt ion and the nucleotide loop with a 5′,6′-dimethyl-benzimidazole base (Bzm) coordinated to cobalt at the lower axial position (α-site). Many B_{12}-producing microorganisms also synthesise different B_{12}-analogues, which have missing or/and substituted elements in the core structure [1,2]. The most typical natural analogues are (i) intermediate products of Cbl synthesis, e.g. a baseless precursor cobinamide (Cbi); and (ii) the complete corrinoids with a nucleotide base differing from Bzm.

Cobalamins with the correct backbone may contain different active groups coordinated to cobalt at the upper

Abbreviations: ATZ, amino-tetrazole; Bzm, 5′,6′-dimethyl-benzimidazole base; Cbl, cobalamin; Ado·/Me·/H_2O·/CN · Cbl, cob(III)alamins with β-coordinated 5′-deoxy-5′-adenosyl, methyl, water, cyanide, respectively; Cbi, cobinamide; GSH, reduced glutathione; His, histidine; Im, imidazole; P_i, sodium-phosphate buffer; RTZ, 1-substituted tetrazole; STZ, Sepharose with tetrazole groups; TZ, tetrazole.

* Corresponding author.
E-mail address: snf@mb.au.dk (S.N. Fedosov).

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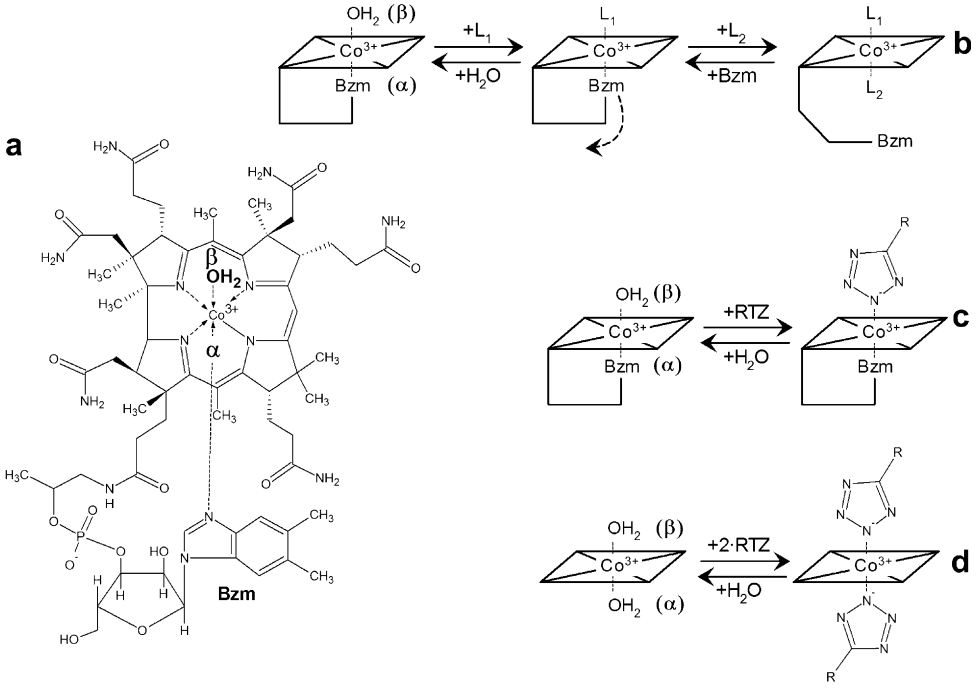

Fig. 1. Structure of $H_2O \cdot Cbl$ and its axial coordinations. (a) Structure of $H_2O \cdot Cbl$ where the Bzm-base and water are coordinated to the lower (α) and upper (β) axial positions, respectively. (b) Displacement of the original axial ligands of $H_2O \cdot Cbl$ by external ligands L_1 and L_2. (c) Coordination of a tetrazole derivative (RTZ) to the upper position of $H_2O \cdot Cbl$. (d) Coordination of two tetrazole derivatives (RTZ) to diaquo-cobinamide.

axial position (β-site): water, cyanide, histidine, etc. [1,2]. Yet, all variants of Cbl can be converted inside the animal cell to the cofactors methyl- and 5′-deoxy-5′-adenosyl-cobalamin ($Me \cdot Cbl$, $Ado \cdot Cbl$), which catalyse reactions of methylation and isomerization, respectively [5]. The carbon–cobalt bond of the two above cofactors is sensitive to light and chemical environment, and it can be easily cleaved under specific conditions [1,2,5]. As a consequence, $Ado \cdot Cbl$ and $Me \cdot Cbl$ can be converted to $H_2O \cdot Cbl$ in oxygenated aqueous solutions. This makes $H_2O \cdot Cbl$ (vitamin B_{12a}) the third ubiquitous form of natural cobalamins.

Water of $H_2O \cdot Cbl$ is a weakly associated axial ligand which can be readily displaced from the β-site of Cbl by a number of other chemicals, for instance CN^- ($K_d < 10^{-12}$ M), ionized sulfhydryl of cysteine or glutathione ($K_d = 10^{-6}$ M), imidazole ($K_d = 10^{-4}$ M), different amino-groups ($K_d = 10^{-3}$ M) [1]. The ligands with highest affinity can also displace the Bzm-base from its lower α-position and form homologous or heterologous axial pairs, as is schematically shown in Fig. 1b. Various combinations of axial ligands have been obtained for baseless analogues, where α-contacts are simplified by absence of Bzm [1,2]. Affinity of an axial ligand for the corrin ring depends on the nature of the opposite group and the degree of cobalt oxidation [1,2]. In an oxygenated solution $[H_2O \cdot Co^{+3}]Cbl$ is a by far prevailing form, which binds a variety of organic and inorganic substances. On the contrary, corrins with chemically or electrochemically reduced cobalt (Co^{2+}, Co^{1+}) bind axial ligands weaker or do not bind them at all.

Coordinating properties of corrinoids are of potential use for adsorption of $H_2O \cdot Cbl$ from a crude solution. This approach might be advantageous in comparison with an unspecific binding to charcoal or different resins, for example, Amberlite XAD. Yet, the list of known solid materials with Cbl binding properties is limited, and these compounds are not quite adequate to the task. For instance, $H_2O \cdot Cbl$ can be adsorbed on amino-Sepharose [6], but the process is slow and requires high concentrations of the ligand. Several other matrices also contain groups with potential affinity for $H_2O \cdot Cbl$, e.g. imidazole derivatives or SH-groups [1]. However, they are expected to be either weak binders or prone to side reactions.

In the current publication we present tetrazole derivatives as novel compounds with high affinity for $H_2O \cdot$ corrinoids ($Co^{3+/2+}$). The active groups can be easily created inside a solid material according to the described method [7]. The Cbl binding properties of the prepared matrix (STZ) were tested under different conditions and argue for a broad applicability of this adsorbent.

2. Results and discussion

2.1. Binding of amino-tetrazole to $H_2O \cdot Cbl$

Incubation of $H_2O \cdot Cbl$ (Co^{3+}) with amino-tetrazole (ATZ) at pH 7.5 was accompanied by characteristic changes in the absorbance spectrum (Fig. 2a), which were quite similar to those in the mixtures of $H_2O \cdot Cbl$ with imidazole or His [8,9]. Kinetics of the reaction ATZ +

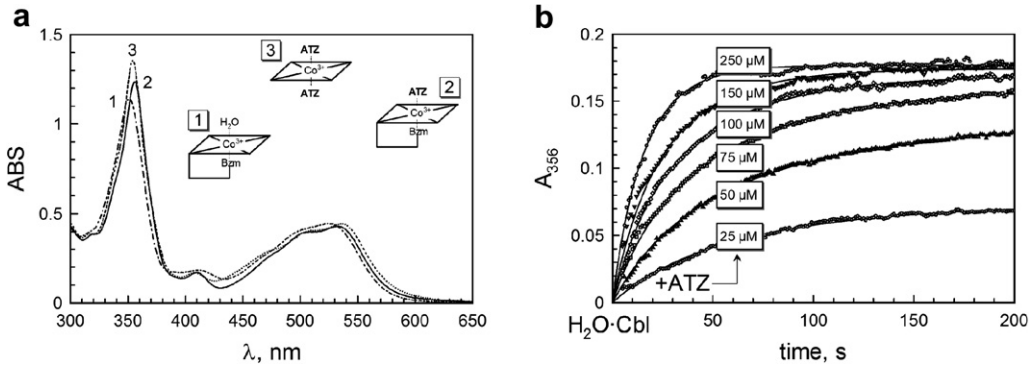

Fig. 2. Change in absorbance spectra upon interactions between ATZ and aquo-corrinoids. (a) Absorbance spectra of 50 μM $H_2O \cdot Cbl$ (1); 50 μM $H_2O \cdot Cbl$ + 2 mM ATZ (2); 50 μM $[H_2O \cdot Co^{3+} \cdot H_2O]Cbi$ + 2 mM ATZ (3). Spectra were recorded in 0.2 M P_i-buffer, pH 7.5, 22 °C. (b) Change in A_{356} followed over time for the reaction $H_2O \cdot Cbl$ (50 μM) + ATZ (25–250 μM), 0.2 M P_i-buffer, pH 7.5, 37 °C.

$H_2O \cdot Cbl \rightleftarrows ATZ \cdot Cbl$ was followed over time by monitoring the changes in absorbance at different ATZ concentrations (Fig. 2b). The association rate constant k_{+ATZ} and the dissociation rate constant k_{-ATZ} were calculated (Table 1). Since the dissociation stability of $ATZ \cdot Cbl$ complex appeared to be quite high $K_d \approx 4$ μM (20–37 °C, pH 7.5, $I = 0.5$ M), the low affinity amino-group was ruled out as a potential coordinating agent. For example, previous analysis revealed a weak binding of amino acids Asp, Lys, Ser to $H_2O \cdot Cbl$ under the same conditions, $K_d \approx 10$ mM [9]. Only histidine was a capable ligand due to presence of imidazole group, $K_d \approx 0.24$ mM [9]. Therefore, the tetrazole moiety of ATZ was suggested to be the component with high affinity for $H_2O \cdot Cbl$ (Fig. 1c).

Nitrogen-containing heterocycles react preferentially with the upper axial position of Cbl [1], scheme in Fig. 1c. Nevertheless, the external ligand present at high concentration can also displace the Bzm-base and coordinate to the lower position, though with reduced affinity and velocity of the binding (Fig. 1b). This binding model was observed in the reaction between $H_2O \cdot Cbl$ and 0.2–1 M imidazole [8], and it cannot be ruled out for ATZ. We did not investigate this subject in further detail, yet the pattern of interaction between $H_2O \cdot Cbl$ and the tetrazole-containing matrix supports the model with two coordination positions. As for the baseless corrinoids, they can easily coordinate the external ligands at both axial positions (Fig. 1d). Indeed, an amplified spectral signal was obtained for diaquo-form of Cbi when mixed with ATZ (Fig. 2a).

The ability of the tetrazole derivatives to bind to $H_2O \cdot Cbl$ is described for the first time. We made a rough estimate of the binding parameters under various conditions. Thus, the reaction was most efficient at pH 4–6 and low ionic strength. However, close spectral properties of $H_2O \cdot Cbl$ and $ATZ \cdot Cbl$ at pH < 7 precluded accurate monitoring of the binding reaction (not shown).

2.2. Preparation of STZ, and kinetics of $H_2O \cdot Cbl$ binding

Attempt to conjugate ATZ via its amino-group to epoxy- or CNBr-activated Sepharose was not particularly successful, and the acquired Cbl-binding capacity did not exceed 0.5–1 mM in the packed matrix. We have therefore changed the strategy. It is known that treatment of organic nitriles with azide is accompanied by formation of tetrazole derivatives, although with different efficiencies [10–12]. The reaction is prompted by the presence of an electron-withdrawing group in close vicinity to nitrile, making the active component of CNBr-activated Sepharose $R–O^{\delta-}–C^{\delta+}{\equiv}N^{\delta-}$ sufficiently promising. The assumed mechanism of the process is shown in Fig. 3a, yet, variable schemes of cyclization are discussed in the literature [11,12]. Incubation of CNBr-activated Sepharose with azide, indeed, led

Table 1
Interaction of NaN_3, ATZ and STZ with $H_2O \cdot Cbl$

Reacting compound	k_+ ($M^{-1}\,s^{-1}$)	k_- (s^{-1})	K_d (μM)	Conditions t (°C), pH, I (M)
NaN_3[a]	2500 ± 150	0.13 ± 0.03	50 ± 10	37, pH 7.5, $I = 0.5$
ATZ	310 ± 20	$12 \pm 4 \times 10^{-4}$	3.9 ± 0.7	37, pH 7.5, $I = 0.5$
	90 ± 10	$4 \pm 1 \times 10^{-4}$	4.4 ± 1.1	22, pH 7.5, $I = 0.5$
STZ	57 ± 3	$6 \pm 1 \times 10^{-4}$	10 ± 2	37, pH 7.5, $I = 0.5$
	20 ± 2	$3 \pm 1 \times 10^{-4}$	15 ± 5	22, pH 7.5, $I = 0.5$
	25 ± 3	$2.2 \pm 0.6 \times 10^{-4}$	9 ± 3	22, pH 6.0, $I = 0.5$
	84 ± 4	$2.0 \pm 0.6 \times 10^{-4}$	2.4 ± 0.7	22, pH 6.0, $I = 0.01$
	Equilibrium (24 h)		≈1.5	22, pH 6.0, $I = 0.5$
	Equilibrium (24 h)		≈0.3	22, pH 6.0, $I = 0.01$

[a] Data from [9].

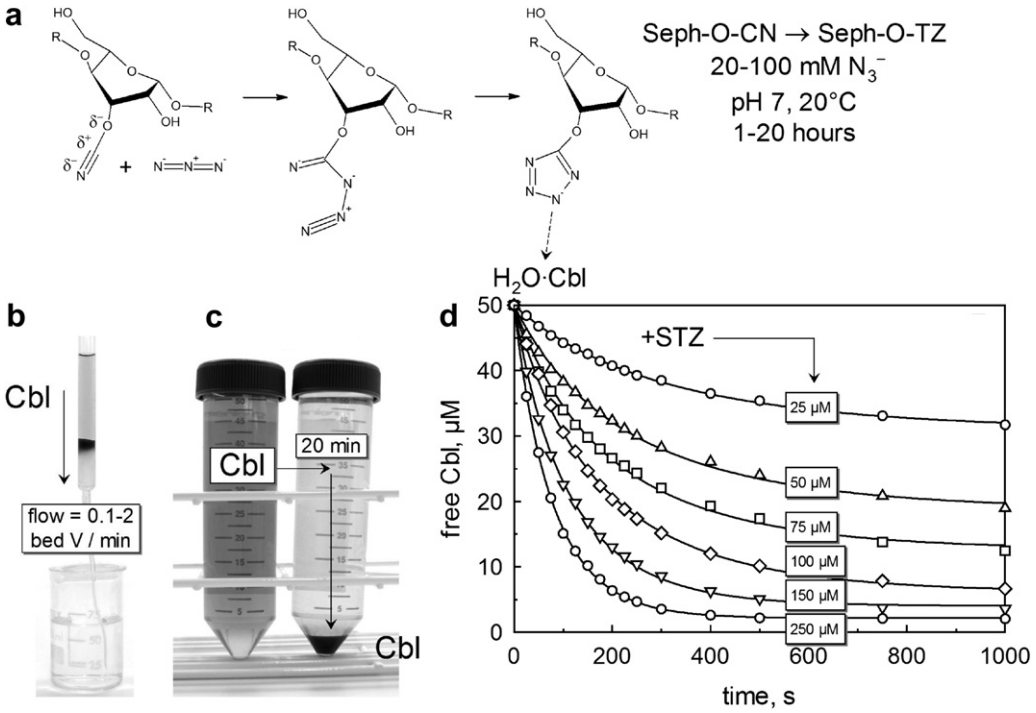

Fig. 3. Adsorption of $H_2O \cdot Cbl$ on STZ. (a) Treatment of CNBr-activated Sepharose with azide, and initiation of tetrazole-groups in the matrix. (b) Filtration of 50 μM $H_2O \cdot Cbl$ through an STZ-column. (c) Batch adsorption of 50 μM $H_2O \cdot Cbl$ on STZ. (d) Kinetics of $H_2O \cdot Cbl$ adsorption on STZ at different concentrations of the binding groups, 37 °C, pH 7.5, $I = 0.5$ M.

to initiation of $H_2O \cdot Cbl$ binding properties in the matrix. Thus, incubation with 20 mM NaN_3 (pH 7–9, 22 °C) gave the following concentrations of the binding groups in the packed material: 7 mM (after 1 h incubation), 8.5 mM (2 h), 9.5 mM (20 h). Higher concentration of azide (100 mM) was slightly better in terms of velocity: 9 mM (1 h), 10 mM (20 h). Increased temperature 37–65 °C accelerated the reaction, so that it was accomplished in 30–60 min. The maximal concentration of the $H_2O \cdot Cbl$ binding groups inside the adsorbent corresponded to 10–12 mM when using fresh CNBr-activated Sepharose.

The prepared Sepharose-tetrazole matrix (STZ) bound $H_2O \cdot Cbl$ in both column chromatography (Fig. 3b) and batch adsorption experiments (Fig. 3c). The binding reaction for $STZ + H_2O \cdot Cbl \rightleftarrows STZ \cdot Cbl$ was investigated under the same conditions as for ATZ (this publication) and azide [9] for comparative purposes. The process was followed by decrease in the concentration of free $H_2O \cdot Cbl$ when incubated in batch at different dilutions of the matrix (Fig. 3d). The calculated rate coefficients showed similarity to ATZ-binding rather than azide-reaction (Table 1) and resulted in $K_d \approx 10$ μM.

2.3. Binding of $H_2O \cdot Cbl$ to STZ under different conditions

The binding conditions under the initial tests (0.2 M P_i-buffer, pH 7.5) were not optimal, see Section 2.1. Therefore, the below experiments address the adsorption of $H_2O \cdot Cbl$ on STZ at different pH, ionic strength and temperature. The pH dependence in Fig. 4a reveals that the binding velocity is influenced by protonation of at least three groups and reaches its maximum at pH 4–7. Two acidic groups are expected to reflect the acid–base equilibria of tetrazole. Protonation of two neighbouring tetrazole-groups with $pK_1 = 0.6$ and $pK_2 = 3.6$ seems to be a more probable explanation than a two step protonation of one residue $STZ^- \rightleftarrows STZH \rightleftarrows STZH_2^+$ according to the known pK values of different tetrazole derivatives [13]. The third equilibrium at alkaline pH ($pK_3 = 8.2$) reflects conversion between aquo- and hydroxo-forms of Cbl [1], where hydroxide ion has higher affinity for cobalt-ion and hinders coordination of tetrazole.

Equilibration of $STZ + H_2O \cdot Cbl$ suspension over a period of time revealed gradual improvement in the binding strength. Thus, approximately a tenfold decrease in the apparent equilibrium constant K_d was observed after 20 h of incubation (Fig. 4b). The effect was detected at different concentrations of NaCl in the mixture and, therefore, could not be ascribed to the presence of a Cbl-coordinating contaminant in the salt. The raise in the affinity is likely to be achieved via formation of the additional STZ–Cbl coordination bond, where the second TZ-group substitutes for the Bzm-base of Cbl as shown in Fig. 4b. The ability of nitrogen-containing heterocycles to displace Bzm from its α-position was observed, for example, at high concentrations of imidazole-derivatives [8]. The estimated value of dissociation constant after complete equilibration of the STZ–Cbl suspension was less than 1 μM.

The data in Fig. 4b also suggest importance of ionic strength for the affinity. Therefore, the dependencies of

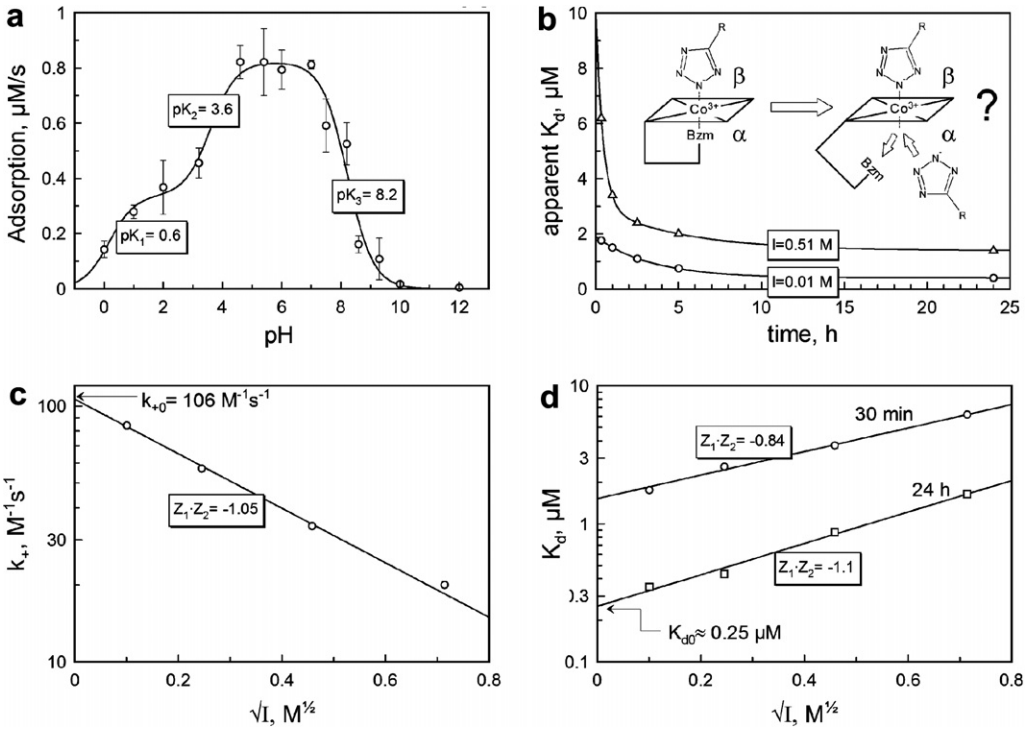

Fig. 4. Interaction between $H_2O \cdot Cbl$ and STZ under different conditions. (a) Velocity of the binding reaction $H_2O \cdot Cbl + STZ$ as function of pH: $Cbl = 30\ \mu M$, $STZ = 400\ \mu M$, 0.1 M phosphate, acetate and carbonate buffers, 22 °C. (b) Change of the apparent dissociation constant ($STZ + H_2O \cdot Cbl \rightleftarrows STZ \cdot Cbl$) over time: 0.01 M P_i-buffer, pH 6.0, ±0.5 M NaCl, 22 °C. (c) Dependence of the attachment rate constant ($STZ + H_2O \cdot Cbl \rightarrow$) on ionic strength: 0.01 M P_i-buffer, pH 6.0, +0.0/0.05/0.2/0.5 M NaCl, 22 °C. Data were fitted according to the equation $\lg(k_+) = \lg(k_0) + 0.509 \cdot Z_1 \cdot Z_2 \cdot \sqrt{I}$. (d) Dependence of the apparent dissociation constant on ionic strength, conditions as in panel C. Measurements were conducted after 30 min and 24 h of incubation. Data were fitted according to the equation $\lg(K_d) = \lg(K_0) - 0.509 \cdot Z_1 \cdot Z_2 \cdot \sqrt{I}$.

the attachment rate constant k_+ and the apparent equilibrium dissociation constant K_d on $I^{1/2}$ were analysed according to the Debye–Hückel's limited law (Fig. 4c and d). Fit of both data sets resulted in the charge product for the interacting molecules ($Z_1 \cdot Z_2$) close to −1 (pH 6, 22 °C). This result corroborates the values of $Z_{Cbl} \approx +1$ and $Z_{TZ} \approx -1$, expected under the experimental conditions (pH 6). Extrapolation to $I = 0$ M gave $k_{+0} \approx 100\ M^{-1}\ s^{-1}$ for the forward reaction, and $K_{d0} \approx 0.25\ \mu M$ for the equilibrated sample (Fig. 4c and d).

Change of the temperature from 22 °C to 37 °C increased velocity of $H_2O \cdot Cbl$ adsorption (Table 1), and the ratio between two rate coefficients $k_{+(37)}/k_{+(22)} \approx 3$ corresponded to the activation energy of $E_a \approx 56\ kJ\ mol^{-1}$. However, the affinity was hardly affected, at least inside the above range of temperature (Table 1).

The performed analysis allowed us to evaluate the velocity of adsorption when a solution of $H_2O \cdot Cbl$ is filtered through a packed column with approximately 10 mM concentration of the binding groups. Under the optimal conditions (pH 4–7, $I \approx 0$) one can expect >99% binding of the ligand in 10 s (22 °C). At higher ionic strength ($I \approx 0.5$ M) this time increases to 50 s. Change of temperature from 22 °C to 50 °C would accelerate the binding by factor ≈8, whereas at 5 °C the adsorption velocity would be four-fold slower than at 22 °C.

2.4. Interaction of different corrinoids with STZ

We have tested interaction of different corrinoids with STZ. The highest affinity was detected for diaquo-form of cobinamide $[H_2O \cdot Co^{3+} \cdot H_2O]Cbi$ ($K_d < 0.1\ \mu M$ at pH 6, $I = 0.01$–0.2 M). Yet, velocity of its attachment was not higher than that for $H_2O \cdot Cbl$, clearly indicating slower dissociation of STZ–Cbi complex. Coordination of two TZ-groups at the upper and lower surfaces of Cbi may account for better retention of this baseless ligand (Fig. 1d). The matrix also bound a number of other aquo-corrinoids: $[CN \cdot Co^{3+} \cdot H_2O]Cbi$, $[H_2O \cdot Co^{3+}]$-pseudo-B_{12}, $[H_2O \cdot Co^{3+}]$-factor A, as well as the reduced forms $[Co^{2+}]Cbi$ and $[Co^{2+}]Cbl$. Parameters of these reactions were not investigated in details, yet, the column adsorption of the mentioned analogues seemed to be as efficient as that of $H_2O \cdot Cbl$.

In contrast to the above Cbl-analogues, there was practically no interaction between STZ and the corrinoids with protected corrin ring, e.g. $CN \cdot Cbl$, $[CN \cdot Co^{3+} \cdot CN]Cbi$, adenosyl-forms of Cbl/pseudo-B_{12}/factor A. The two latter substances presented an interesting example of trans-axial effect, where the ligand coordinated to one side of the ring affected interactions at the opposite side via the cobalt ion. It is known that the efficient donation of electrons from the upper Ado-group to the Co-ion causes detachment of the nucleotide base from the lower surface of pseudo-B_{12} and

factor A [2,14]. It seems that the binding of tetrazole in place of the dissociated nucleotide was also precluded.

Apart from the above groups with high affinity for the cobalt-ion, the axial compounds with moderate binding strength could also negatively affect STZ + Cbl interactions. The group of adverse effectors includes, for example, 1 mM glutathione (GSH), 10 mM imidazole, 1 mM azide, 100 mM NH_4OH, pH ≈ 11. Several binding curves for protected corrinoids are presented in Fig. 5a.

2.5. De-blocking of the β-protected Cbl

Depending on the properties of the protecting group, de-blocking of the β-site can be done in different ways, and below we mention several general approaches to the problem (Fig. 5b). The most resistant axial ligand is cyanide, and conversion of CN · Cbl to H_2O · Cbl requires chemical or electrochemical reduction followed by oxidation in aqueous solution [1,2]. The carbon–cobalt bond of the two natural organo-cobalamins Ado · Cbl and Me · Cbl is sensitive to light, and illumination of the sample for 15–30 min in aerated solution would produce H_2O · Cbl [1,9]. The same is also valid for other organo-corrinoids.

Many axial ligands with moderate affinity for the corrin ring can be dissociated at low pH, because they essentially lose Cbl-binding properties after protonation, e.g. GS · Cbl ⇄ GS^- + Cbl ⇄ GSH + Cbl. Application of other de-blocking agents is also possible. For instance, dissociation of glutathione is facilitated by Cu^{2+} due to the complexation reaction $GS^- + Cu^{2+}$ ⇄ $Cu(GS)_2$. This effect was observed at 1 mM GSH in the presence of 2 mM $CuSO_4$, pH 3–4. As a universal approach to the specific binding of H_2O · Cbl from various solutions with hindering compounds we can suggest adsorption on the STZ at pH 3–4. These conditions are not optimal, however the column filtration at a reduced flow (0.1–0.2 bed volume per minute) makes adsorption of H_2O · Cbl quite efficient. All the mentioned methods of Cbl-reactivation are schematically depicted in Fig. 5b.

2.6. Other hindering compounds

Cbl binding proteins with high affinity for the ligand belong to the group of substances, which affect H_2O · Cbl adsorption on STZ [3,9,15,16]. Thus, attachment of H_2O · Cbl to the specific transporting proteins or enzymes precluded any interaction with the matrix. Most proteins can be however denatured by heating at 85–90 °C, pH 3.5, 15 min.

Transition metal ions M^{2+} (Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) can potentially decrease affinity of STZ for H_2O · Cbl by direct coordination to the tetrazole groups STZ · M^{2+} [17]. Indeed, presence of 1 mM $CuSO_4$ in the medium (pH 3.5) decreased velocity of the binding H_2O · Cbl + STZ by factor 5 (not shown). However, the mentioned ions (M^{2+}) can be removed from the solution by adjustment of pH > 7 and precipitation of the insoluble metal hydroxide $M(OH)_2$.

2.7. Elution of the bound corrinoids from STZ

Retention of the adsorbed Cbl by STZ at pH 4–7 was very efficient if the saturation level was below 80%. Thus, no significant leakage of the ligand was detected during the column washing using solutions with different ionic strength (0.01–2 M) and temperature (20–65 °C). However, a progressively increasing desorption was observed at higher degree of saturation (80–100%).

Liberation of Cbl from the affinity adsorbent can be carried out in several ways depending on the purpose of the work. Batch mode (STZ:eluent = 1:10), when using a warm alkaline buffer with added KCN (e.g. pH 12, KCN = 10 mM, 95 °C, 5–10 min), results in fast and quantitative elution of Cbl in its dicyano-form. As high temperature at pH 12 can adversely influence chemical stability of Sepharose, we recommend the above method only for the analytical purposes, e.g. measurement of the titer of the active TZ-groups (see Section 4). Usage of lower temperatures is gentler to the matrix and allows its repeated appli-

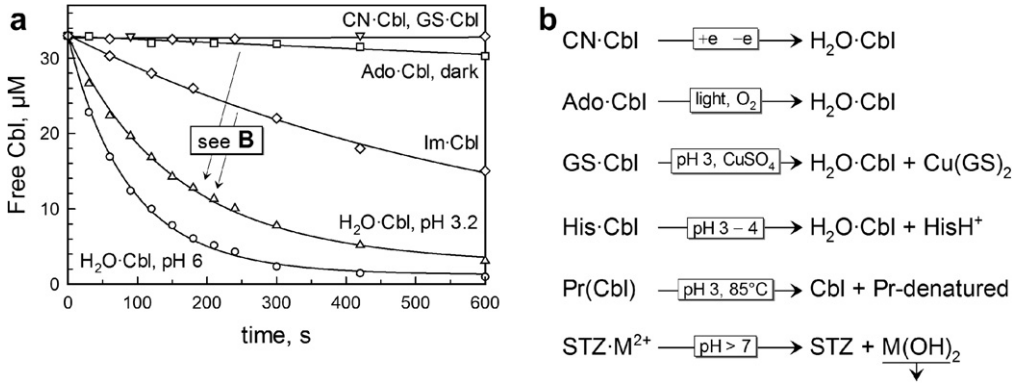

Fig. 5. Hindering effect of different compounds on the binding of Cbl to STZ. (a) Adsorption curves for different cobalamins: H_2O · Cbl; Im · Cbl (H_2O · Cbl + 10 mM imidazole); Ado · Cbl; CN · Cbl; GS · Cbl (H_2O · Cbl + 1 mM GSH); 0.01 M P_i-buffer, pH 6, 22 °C. (b) Conversion of the STZ-inert cobalamins to H_2O · Cbl. Notation: CN·/Ado·/GS·/His · Cbl correspond to cyano-/adenosyl-/glutathionyl-/histidine-forms of Cbl; Pr(Cbl) represents protein-bound Cbl; STZ · M^{2+} is a complex between the tetrazole matrix and a transition metal ion.

cation. For example, 98–100% liberation of dicyano-Cbl could be achieved at room temperature after 5–10 h of incubation in 10 mM KCN, pH 9–12. If necessary, dicyano-Cbl can be converted to mono-cyano form CN · Cbl by acidification of the medium to pH < 4 or removal of excessive cyanide.

Desorption from an STZ-column required longer time of incubation (20–40 h, 20–25 °C) and higher concentrations of eluent (e.g. 20 mM KCN, 0.1 M P_i-buffer, pH 12) when compared with batch elution. One change of the eluent in the column was required after 10–20 h to guarantee ≈95% liberation of the bound Cbl in its dicyano-form. Elution at lower pH was also possible (8–9), but with decreased efficiency of desorption. For example, 2–3 changes of the medium were required at pH 9 to reach the same yield as at pH 12. Other corrinoids can be liberated in similar way. Desalting of the obtained preparations can be done on XAD or charcoal, see Section 4. The final product would be CN · Cbl.

Two alternative methods of elution are based on pH-properties of the binding material, where acidic or alkaline medium hampers the binding (Fig. 4a). Thus, H_2O · Cbl can be eluted from a column in 10 min using 2–3 bed volumes of 1 M HCl at continuous flow. The collected sample should be neutralized to minimize slow hydrolysis of the amide side chains in the strong acid [1]. Desorption in an alkaline medium (e.g. 0.1 M NaOH) was slower (10–20 h) and required one change of the solution. The product (HO · Cbl) contained 10–20% of contaminations which could originate from either self-reduction of HO · Cbl in alkaline medium or displacement of HO^- from the β-site by some other compounds [1]. This subject was not investigated further. It should be emphasized that the pH-based methods were inefficient in elution of the baseless analogue [$H_2O \cdot Co^{3+} \cdot H_2O$]Cbi where both axial position seemed to be occupied by the tetrazole groups of STZ (Fig. 1d). Therefore, H_2O · Cbl and [$H_2O \cdot Co^{3+} \cdot H_2O$]Cbi could be separated by adsorption on STZ-column and sequential elution with (i) 1 M HCl (H_2O · Cbl); and (ii) 20 mM KCN, pH 12 (dicyano-Cbi).

2.8. Chemical and physical stability of the STZ

The chemical and physical stability of the adsorbent was sufficiently high. Thus, no deterioration of the binding properties was observed after a year of incubation in water at room temperature or after several months in 0.1 M P_i-buffer, pH 12, 10 mM KCN. Neither 1 M HCl nor 0.1 M NaOH immediately affected the Cbl binding properties of the material. However, physical stability of the matrix against pressure was noticeably deteriorated under strong alkaline conditions, and the Sepharose particles could be easily mashed in 0.1 M NaOH under gravity forces, e.g. in a column of 50 cm height. Prolonged incubation of STZ in acid gradually decreased the Cbl-binding capacity of the material, and after 50 h of incubation in 1 M HCl the maximal saturation of STZ drops by 30%. No such effect was found for 0.1 M NaOH, where two months incubation in a column of 2 cm height did not decrease the level of Cbl adsorption.

2.9. Purification of Cbl from a crude mixture

We tested application of the STZ on a fermentation broth provided by a manufacturer of vitamin B_{12}. A standard acidic extraction of Cbl from cells was conducted in the absence of cyanide. The binding from extract was conducted under suboptimal conditions (pH 3.7) since the contaminating substances (possibly reduced glutathione) precluded coordination of Cbl to STZ at pH 6. Adsorption was conducted in two cycles, preceded by illumination of the extract. Approximately 70% and 20% of the total Cbl was adsorbed from the medium after the first and second passages, respectively. The levels of the bound Cbl were independent of the flow rate at $v = 1$–6 bed volumes per hour. On the contrary, the illumination of the sample was critical, since only 30% of Cbl was adsorbed after the first filtration in darkness. Finally, the column material was saturated with Cbl to the level of 7–8 mM in the packed matrix. The succeeding purification procedure included following steps: (1) washing with 0.5 NaCl and water; (2) elution with cyanide at alkaline pH; (3) desalting on either XAD or charcoal. The latter procedure removed also excess of cyanide and converted dicyano-Cbl to CN · Cbl. The absorbance spectrum of the final preparation in water was indistinguishable from that of commercial CN · Cbl, and HPLC analysis pointed to >90% purity of the sample. The final yield corresponded to 80% of Cbl present in the original fermentation broth.

2.10. Application of STZ for measurement of Cbl-binding capacity and dissociation kinetics

Association of H_2O · Cbl with proteins hinders adsorption on STZ. This property can be advantageously used to measure the Cbl binding capacity of such protein by spectral method. Firstly, the protein should be saturated with some excess of H_2O · Cbl, whereupon the unbound ligand should be removed by adsorption on STZ. Finally, the absorbance spectrum of the protein-bound Cbl can be measured and used to calculate concentration of this complex. Details of this approach are described elsewhere [15].

Ability of STZ to remove free H_2O · Cbl from soluble phase can be used to study dissociation kinetics of different complexes, where H_2O · Cbl is bound to natural or synthetic compounds with moderate affinity, e.g. the fragments of intrinsic factor [15]. In these experiments, excess of the TZ-groups caused gradual dissociation of H_2O · Cbl-fragment complexes and allowed us to measure the corresponding rate constants [15].

3. Conclusions

A simple treatment of CNBr-activated Sepharose with azide produces the matrix with 10 mM concentration of the active groups (presumably tetrazole), which have high affinity and specificity for aquo-corrinoids. Efficient binding can be conducted in a broad range of pH, ionic strength and temperature. Adsorption from a crude cell extract is also possible. Presence of certain contaminants hampers the binding, yet, the effect can be neutralized by changing conditions of adsorption. The novel material is applicable for purification and separation of different corrinoids, as well as for analytical purposes, e.g. investigation of protein–corrinoid interactions. At the same time, the soft Sepharose-based adsorbent does not seem to be adequate for industrial purposes. As an alternative, a robust Cbl-specific polymer could be synthesized, where nitrile-containing monomeric compounds are treated with azide and afterwards polymerized.

4. Experimental

4.1. Materials

All salts and simple organic materials were from Sigma–Aldrich and Merck. Cobalamins $H_2O \cdot Cbl$, $CN \cdot Cbl$, $Ado \cdot CBl$, dicyano-Cbi were from Sigma–Aldrich. Ado-forms of pseudo-B_{12} and factor A were kindly provided by Prof. B. Kräutler [14]. Conversion of pseudo-B_{12} and factor A to their aquo-forms was performed by illumination (60 W lamp, 10 cm distance, 10 min). Cobinamides $[CN \cdot Co^{3+} \cdot H_2O]$Cbi and $[H_2O \cdot Co^{3+} \cdot H_2O]$Cbi were synthesized from $CN \cdot Cbl$ and $H_2O \cdot Cbl$, respectively [18]. Reduced forms $[Co^{2+}]$ of Cbl and Cbi were obtained from $[H_2O \cdot Co^{3+}]$Cbl and $[H_2O \cdot Co^{3+} \cdot H_2O]$Cbi, respectively, by treating them with 15% formaldehyde, 5 min, 95 °C.

4.2. Kinetics of interaction between ATZ and $H_2O \cdot Cbl$

Solution of 50 μM $H_2O \cdot Cbl$ in 0.2 M P_i-buffer, pH 7.5 (22 °C or 37 °C) was mixed with different concentrations of ATZ = 25–250 μM, whereupon change in absorbance was followed at 356 nm. Caution: any contacts between ATZ and metals (including Hamilton Microliter syringes) must be avoided. The recorded curves in Fig. 2b were analysed by appropriate equations of reversible kinetics $A + B \rightleftarrows C$ as explained elsewhere [15].

4.3. Preparation of STZ

Both Sepharose 4B (Amersham) activated by CNBr under laboratory conditions and the commercially available equivalent product (Amersham) can be used for the preparation of the Cbl binding matrix. Laboratory treatment of Sepharose with CNBr resulted in 30–50% higher level of the binding groups and was essentially cheaper. The procedure is presented below.

Hundred milliliter of Sepharose 4B Fast Flow was placed into an open thick column ($d = 5$ cm, $h = 40$ cm) capable of fast flow filtration. The matrix was washed with 400 mL water and 200 mL of 2 M K_2HPO_4/K_3PO_4, pH 11, until no liquid remained above the packed Sepharose. The flow was stopped, the column was placed into a fume hood, and 100 mL of 2 M phosphate buffer, pH 11, was added to 100 mL of Sepharose to make slurry 1:1. The reaction was started by adding freshly prepared 10% CNBr dissolved in 250 mL H_2O at 40–50 °C and cooled to room temperature. The CNBr solution (22 °C) was added to the Sepharose suspension in three portions at 0, 1, 2 min of the reaction under continuous mixing. The process was carried on for another 3 min while pH fell to pH 8. Then, the reaction suspension was drained (2–3 min), and the CNBr-activated Sepharose was washed with 100 mL of 0.1 M P_i-buffer, pH 7.5; 500 mL H_2O; and again 100 mL of P_i-buffer. The flow was stopped when no liquid remained above the packed matrix. Immediately afterwards the prepared material was interacted with azide to produce STZ.

Reaction between the nitrile groups of Sepharose and azide was started by adding 100 mL of 100 mM NaN_3 dissolved in 0.1 M P_i-buffer, pH 7.5 (50 mM final concentration of NaN_3 in the slurry). The suspension was periodically agitated for the first 30 min and left either overnight at 22 °C or for 3–4 h at 37 °C. Next day STZ was washed with 200 mL of 0.1 M Pottassium-phosphate buffer, pH 11.5–12, and incubated either overnight at 22 °C or for 3–4 h at 37 °C. The latter alkaline treatment increased the amount of Cbl-binding groups by 50–70%. Finally, the prepared material was washed with 1 L of H_2O, and a slurry STZ:H_2O = 1:1 was prepared. STZ can be stored both at room temperature and at 5 °C at least for a year without any deterioration of the Cbl-binding properties.

If commercially available CNBr-activated Sepharose 4B is used for preparation of STZ, it should be extensively washed for 15–20 min with 1 mM HCl and then equilibrated with a neutral solution of 50 mM NaN_3 as described in the above paragraph.

4.4. Measurement of the active groups in STZ

0.05 ml of the suspension STZ:water (1:1) was mixed with 0.5 ml of 1 mM $Cbl–OH_2$ in 0.01 M P_i-buffer, pH 6 and incubated for 5 min. The sample was centrifuged (10 000 rpm, 30 s), the supernatant was discarded, and the coloured pellet was washed with 2 ml of 0.1 M P_i-buffer, pH 6 for 1 min. The adsorbent was collected by brief centrifugation, and the bound Cbl was eluted in 5 min under the following conditions: 2 ml of 2 mM KCN, 0.1 K_2HPO_4/K_3PO_4 buffer, pH 12, 95 °C. The concentration of the eluted dicyano-Cbl was measured in the supernatant according to the following absorbance coefficients, $\varepsilon_{367} = 30\,400$, $\varepsilon_{579} = 9900\ M^{-1}\ cm^{-1}$. The concentration of the Cbl-binding groups in the original STZ (25 μL) is expected to be 8–12 mM, which corresponds to 4–6 mM in the slurry STZ:water = 1:1.

4.5. Kinetics of interaction between STZ and $H_2O \cdot Cbl$

Solutions of 50 μM $H_2O \cdot Cbl$ in 0.2 M P_i-buffer, pH 7.5 (22 °C or 37 °C) were mixed with different amounts of STZ, e.g. a 10 mL sample plus 0.050–0.5 mL suspension of STZ:water (1:1), final concentration of STZ = 25–250 μM. The prepared mixtures were incubated with slight agitation, and 0.5 ml fractions were filtered under pressure after different time intervals. The concentration of remaining $H_2O \cdot Cbl$ in the filtrates was measured ($\varepsilon_{352} = 23\,200\ M^{-1}\,cm^{-1}$) and plotted over time (Fig. 3a). Analysis of bimolecular binding kinetics was performed as described elsewhere [15].

The binding experiments for STZ plus an aquo-corrinoid (Cbl, Cbi, pseudo-B_{12}, factor A) were conducted in similar way at varying pH, ionic strength and temperature. The following absorbance coefficients ($M^{-1}\,cm^{-1}$) were used for $H_2O \cdot Cbl$: $\varepsilon_{351} = 26\,200$ (pH 2–5); $\varepsilon_{352} = 23\,200$ (pH 7.5); $\varepsilon_{358} = 20\,500$ (pH 10–12).

An apparent equilibrium dissociation constant for the reaction STZ + Cbl ⇄ STZ · Cbl was measured in the mixtures of the following composition: $H_2O \cdot Cbl = 33$ μM, STZ = 50 μM,

$$K_d = \frac{[Cbl] \cdot [STZ]}{[STZ \cdot Cbl]}; \quad [STZ] = [STZ]_0 - [STZ \cdot Cbl]; \quad [STZ \cdot Cbl] = [Cbl]_0 - [Cbl]$$

0.01 M P_i-buffer, pH 6.0 ($I \approx 0.01$ M), NaCl = 0.0–0.5 M, 22 °C. Concentrations of free $H_2O \cdot Cbl$ ([Cbl]) were measured at time intervals in the supernatant using the absorbance coefficient $\varepsilon_{351} = 25\,500\ M^{-1}\,cm^{-1}$. The value of K_d was calculated according to the above equation.

The dependences of K_d on time (Fig. 4b) and ionic strength (Fig. 4d) were plotted and analysed as explained in the main text.

The equilibrium dissociation constant was measured by incubation of 20 μM $H_2O \cdot Cbl$ with different dilutions of STZ (0–80 μM) for 24 h (22 °C). The analysis of the binding curve [STZ · Cbl] vs. $[STZ]_{total}$ was conducted using the square root equation [15].

4.6. Purification of Cbl from cell extract

Genetically improved cell line of *Pseudomonas denitgrificans* was used for production of cobalamin, see Ref. [19] as a review. Cell extract was prepared by a manufacturer of B_{12} by adding H_2SO_4 to the fermentation broth (final pH 3.7) and heating it for 15 min at 85 °C. No cyanide was present to avoid conversion of natural cobalamins to CN · Cbl. Concentration of cobalamin in the extract (320 μM) was measured spectroscopically after HPLC fractionation of a cyanide treated sample. The bulk of extract (5 L) was centrifuged and used for adsorption on the STZ-column ($d = 5$ cm, $h = 12$ cm, ≈240 mL). Adsorption on STZ was carried out in two cycles (20–24 h) with illumination of the extract at the entrance to the column. The illumination unit consisted of a 60 W lamp placed at the distance of 10 cm from a looped silicon tubing, $l = 2$ m, $d = 0.5$ cm, Buch and Holm (Denmark). Other lamps and tubings can be used as well. The column with adsorbed Cbl was sequentially washed with H_2O (0.5 L), 0.5 M NaCl (0.5 L), H_2O (1.5 L), and equilibrated with approximately 200 mL of 20 mM KCN, 0.1 M K_2HPO_4/K_3PO_4, pH 12, until a noticeable leakage of Cbl started. Elution was conducted in steps, where 100 mL fractions were eluted after 20 h, +6 h and +20 h of incubation (22 °C).

Desalting, removal of excessive cyanide and additional purification of the obtained sample was carried out in a 300 mL column packed with Amberlite XAD-2. The sample from STZ (300 mL, [Cbl] ≈ 5 mM) was loaded on XAD in three steps with 8–20 h incubation between each step. The column was washed with 3 × 100 mL of H_2O (3 × 30 min) and 3 × 100 mL of 5 mM acetate pH 3.5 (3 × 30 min). Elution was conducted with 3 × 100 mL of 20% 2-butanol (3 × 30 min), whereupon CN · Cbl solution was lyophilized.

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