A cyclodecapeptide ligand to vitamin B_{12}†

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Libraries of cyclic decapeptides were screened with vitamin B_{12} derivatives to give cyclic peptide ligands incorporating histidine and cysteine as coordinating residues and negatively charged amino acids. Two hits, cyclo-(HisAspGluProGlyIleAlaThrProDGln) and cyclo-(ValAspGluProGlyGluAspCysProDGln) were resynthesized in good yields for solution experiments. The peptides bind aquocobalamin with coordination of His or Cys to the cobalt with high affinities (K_a ~10^5 M^{-1}). Additional interactions between the peptide side chains and the vitamin B_{12} corrin moiety were determined by studying the ^{1}H NMR solution structure. The cyclopeptide–cobalamin complex with the histidine residue showed enhanced stability towards cyanide exchange, demonstrating the shielding effect of the ligand on the metal center.

Introduction

Proteins binding to porphyrin-type cofactors create a large shell around them that affects their reactivity and stability for transport and catalysis. Coordination of these cofactors to smaller peptide-based models of these proteins provides useful systems to study their essential properties. While many such model-systems have been reported based on linear peptides[1] and dendrimers,[2] there are only very few studies of cyclic peptide ligands, which only concern heme so far.[3] Herein we report the first cyclic peptide complexes of vitamin B_{12}, a corrin-containing cofactor bound *in vivo* to transport proteins and enzymes.[4] The complexes show enhanced stability compared to small molecule analogs and a reduced reactivity towards nucleophiles, as observed for related dendritic peptide ligands.[5] The ^{1}H NMR-structure of the complex shows a cobalt–cysteine residue bond and additional multiple contacts between the cyclodecapeptide and the corrin ring. This cyclic peptide features a remarkably simple and well-defined model of B_{12}-binding proteins. Peptide-based ligands for vitamin B_{12}, such as the cyclodecapeptides reported here, might find application as adjuvants for B_{12}-formulations for facilitating uptake by patients deficient in this intrinsic factor.[6]

Results and discussion

Ligand library design and synthesis

Cyclic decapeptides incorporating a pair of β-turn inducing Pro-Gly dipeptides display a preorganized structure originally designed as an anchoring point for the attachment of further peptides to assemble so-called RAFT-proteins.[7] We considered the recently reported solid-supported RAFT synthesis starting with side-chain attachment at a D-glutamate residue[8] for the solid-phase synthesis of a split-and-mix combinatorial library.[9] The D-glutamate residue at position X^1 is part of one of the two conserved turn dipeptides (qP at X^1X^2 and GP at X^6X^7), separating two variable tripeptides $X^3X^4X^5$ and $X^8X^9X^{10}$ (see Scheme 1). The amino acids at the variable positions were assigned following our recently reported "unique pair" algorithm called TAGSFREE.[10] This algorithm creates libraries in which peptide sequences can be assigned from the amino acid composition analysis. The method is particularly advantageous for cyclic peptides, which are not amenable to Edman sequencing.[11]

The 15 625 member "unique pair" combinatorial library with 5 amino acids at each of the 6 variable positions was designed for potential B_{12}-binding by coordination of the cobalt with a histidine or cysteine side-chain, by placing these residues at variable positions on either side of the RAFT framework (cysteine at positions X^3 and X^9 and histidine at positions X^8 and X^{10}). The other 13 amino acids allowed by the unique pair design were distributed along the variable positions to cover a variety of charged, hydrophobic, aromatic, small and polar residues.

The library was prepared using Fmoc-chemistry on a 1 g batch of Rapp Polymers TentaGel Macrobeads (0.3 mmol g^{-1}, 1.6 × 10^5 beads per gram) allowing 10-fold coverage of sequence space. The synthesis was completed by removal of the α-*N*-Fmoc group, deallylation of the α-carboxyl group of the D-glutamate at position X^1, and on-bead cyclization using PyBOP–DIEA in DMF (2 × 60 min). In the final step of the synthesis, side-chain protecting groups were removed by acidic treatment, leaving the on-bead library of cyclic decapeptides ready for activity screening.

On-bead binding assay and ligand synthesis

The cyclodecapeptide library was assayed for aquocobalamin binding. A portion of the library (10 mg) was first equilibrated in aqueous PBS (20 mM phosphate, 150 mM NaCl, pH 7.4), and then incubated with the vitamin, followed by extensive washing

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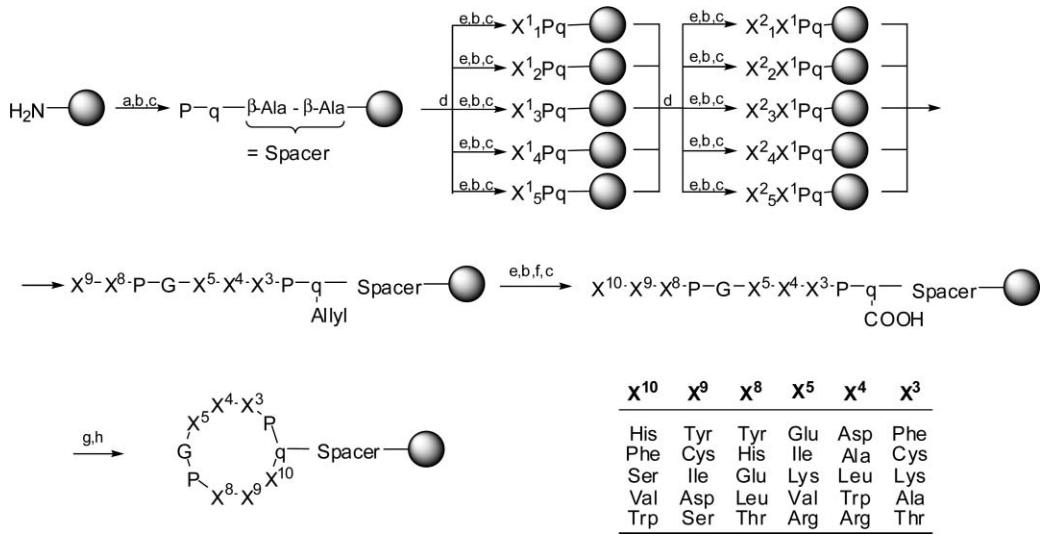
† Electronic supplementary information (ESI) available: Plots of exchange kinetics with cyanide, HPLC traces of bead analysis, tables for non-hits and sequences picked randomly, analytical HPLC profiles and +ESI-MS data. See DOI: 10.1039/b811234g



X^{10}	X^9	X^8	X^5	X^4	X^3
His	Tyr	Tyr	Glu	Asp	Phe
Phe	Cys	His	Ile	Ala	Cys
Ser	Ile	Glu	Lys	Leu	Lys
Val	Asp	Leu	Val	Trp	Ala
Trp	Ser	Thr	Arg	Arg	Thr

Scheme 1 Synthesis and structure of the cyclic peptide library. The solid support is Rapp TentaGel Macrobeads (0.3 mmol g^{-1}, 1.6×10^5 beads per g). Using five α-*N*-Fmoc protected amino acids at positions X^n (n = 3–5, 8–10) gives a library of $5^6 = 15\,625$ members. *Conditions*: (a) 3 eq. Fmoc protected amino acid, 3 eq. PyBOP, 6 eq. DIEA, DMF, 2 × 60 min. (b) Ac_2O–CH_2Cl_2 (1 : 1), 15 min. (c) 20% piperidine in DMF, 3 × 10 min. (d) The resin batch was suspended in DMF–CH_2Cl_2 (2 : 1, v/v), mixed *via* nitrogen bubbling for 15 min, and the batch was split into five equal portions 1–5 by volume. (e) In each portion $X_b{}^a$ (b = 1–5): 3 eq. Fmoc-$X_b{}^a$-OH, 3 eq. PyBOP, 6 eq. DIEA, DMF, 2 × 60 min. (f) $PhSiH_3$, $Pd(PPh_3)_4$, dry CH_2Cl_2, under argon, 2 × 30 min. (g) PyBOP, DIEA, DMF 2 × 45 min (h) TFA–TIS–H_2O–EDT (94 : 1 : 2.5 : 2.5, v/v) 3 h.

and visual detection of stained beads. Under optimized assay conditions (100 μM aquocobalamin in PBS at 25 °C for 2.5 hours), only very few beads remained stained (less than 2%, Fig. 1). Deep red colored beads were picked and subjected to amino acid analysis. From 24 beads, 18 returned a readable sequence (Table 1). While control beads picked at random from the library showed no sequence preference (Table S3 in the ESI†), the B_{12}-stained beads were rich in anionic (Glu at X^5/X^8 and Asp at X^4/X^9) and hydrophobic residues (Leu, Val, Ile). In twelve sequences cysteine residues were present while histidine was found in one sequence as the only possible coordinating residue and in two with cysteine. Five beads gave no coordinating residues but incorporated the anionic residues as well. On the other hand, cationic and aromatic residues were almost absent (Table 1, Table 2). The obtained results indicate the possible coordination of cysteine to cobalt as a binding feature. Coordination to cobalt was also suggested by the absence of hits when the library was screened with cyanocobalamin or methylcobalamin, where the ligands in the upper side can not be easily exchanged.

The cyclodecapeptide sequences **3a**, **3b** with His, **4**, **11** with Cys, **9a** with both coordinating residues and sequence **12** without His or Cys were selected as representative hits from the screening. They were synthesized on Rink-amide resin using on-bead cyclization as above, followed by acidic deprotection and HPLC purification of the cleaved cyclic peptides, providing the material in good yields and purity (Table 3). For comparison, the corresponding linear sequences (**4′** and **4′Ac**) were also synthesized and isolated by HPLC. In addition, we prepared the mutant **4His** of peptide **4** in which the coordinating cysteine residue is replaced by histidine. Although the screening method gave mainly cyclic peptides with cysteine, the His containing ligands better simulate the binding mode of aquocobalamin in the transport protein transcobalamin, where a histidine residue is coordinated on the upper phase of the cofactor.[12]

Table 1 Hits from cyclodecapeptide library $X^{10}X^9X^8$-Pro-Gly-$X^5X^4X^3$-Pro-gln

	X^{10}	X^9	X^8	X^5	X^4	X^3
1	Ser	Asp	Glu	Glu	Arg	Cys
2a[a]	Ser	Tyr	Leu	Glu	Asp	Cys
3a[b]	His	Asp	Glu	Ile	Ala	Thr
4	Val	Asp	Glu	Glu	Asp	Cys
5	Val	Cys	Glu	Glu	Leu	Thr
6a[c]	Val	Ser	Glu	Ile	Asp	Thr
7a[d]	Val	Ser	Leu	Glu	Asp	Ala
8	Val	Asp	Glu	Val	Asp	Thr
9a[e]	His	Cys	Thr	Glu	Leu	Ala
10	Ser	Ile	Glu	Glu	Trp	Cys
11	Val	Ile	Glu	Glu	Asp	Cys
12	Val	Ile	Glu	Glu	Asp	Ala
13a[f]	Val	Asp	His	Glu	Ala	Cys
14a[g]	Val	Asp	Leu	Glu	Ala	Cys
15a[h]	Val	Ser	Leu	Glu	Asp	Cys
16	Val	Ile	Glu	Glu	Leu	Thr
17a[i]	Val	Ser	Tyr	Glu	Ala	Cys
18a[j]	Val	Ile	Leu	Glu	Asp	Cys

Alternative sequences from decoding:[a] SDYEKC; [b] HITEDA (**3b**); [c] SIEVDT; [d] SDEVLA; [e] HCLEAT; [f] HDEVAC, VCHEDA, HCEVDA; [g] VCLEDA; [h] SDEVLC; [i] STEVAC; [j] VDEILC.

Binding affinities to B_{12}

The binding affinities of the synthesized hits **3a**, **3b**, **4**, **9a**, **11** and **12** for aquocobalamin were determined by UV-titration (Fig. 2, Table 4). The cyclic peptides **4** and **11** coordinate the cobalamin with the cysteine residue as evidenced by the UV changes whereas

Table 2 Hit percentage occurrence of the amino acid at the given position: H% (P%), H% is the percentage occurrence of the amino acid at the given position in picked hits; the relative occurrence P% is the observed frequency of the amino acid at the given position across a random analysis of 24 beads (see ESI†)

No.	AA	X^{10}	X^{9}	X^{8}	X^{5}	X^{4}	X^{3}
1	Tyr		6% (36%)	6% (25%)			
2	His	14% (18%)		3% (21%)			0% (21%)
3	Phe	0% (21%)					50% (5%)
4	Cys		17% (20%)				
5	Glu			67% (14%)	72% (14%)		
6	Ile		31% (18%)		8% (38%)		
7	Lys				0% (5%)		0% (25%)
8	Asp		36% (13%)			47% (13%)	
9	Ser	28% (7%)	11% (14%)				
10	Val	58% (21%)			19% (18%)		
11	Ala					17% (5%)	22% (9%)
12	Leu			19% (16%)		25% (32%)	
13	Thr			6% (23%)			28% (39%)
14	Trp	0% (32%)				6% (18%)	
15	Arg				0% (25%)	6% (32%)	

Table 3 Sequences, isolated synthetic yields (mg, %) and +ESI-MS data for the cyclic peptides and the linear controls, including the *N*-acetylated linear and mutant **4His** sequences

Peptide	Structure	Yield/ mg (%)	+ESI-MS (calcd)	+ESI-MS (found)
3a	c-(HDEPGIATPq)	41 (79)	1046.5	1046.6
3b	c-(HITPGEDAPq)	24 (47)	1046.5	1046.4
4	c-(VDEPGEDCPq)	46 (86)	1070.4	1070.6
4His	c-(VDEPGEDHPq)	32 (58)	1104.5	1104.6
4′	$VDEPGEDCPGNH_2$	46 (82)	1016.4	1016.4
4′Ac	$AcVDEPGEDCPGNH_2$	40 (76)	1058.4	1058.4
4His′	$VDEPGEDHPGNH_2$	35 (66)	1050.5	1050.4
4His′Ac	$AcVDEPGEDHPGNH_2$	38 (69)	1092.5	1092.5
9a	c-(HCTPGELAPq)	18 (35)	1034.5	1034.5
11	c-(VIEPGEDCPq)	48 (90)	1068.5	1069.0
12	c-(VIEPGEDAPq)	39 (76)	1036.5	1036.5

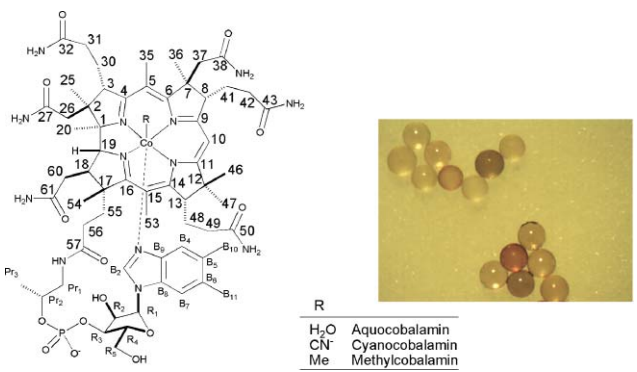


Fig. 1 Structure of vitamin B_{12} derivatives, and on-bead binding assay with aquocobalamin (a portion of the screened batch is seen).

3a and **3b** showed the shifts expected for imidazoyl coordination. Addition of hit **12** did not induce changes in the UV-spectrum of aquocobalamin, showing that cobalt coordination with side chain carboxylic groups is not taking place, although peripheral interactions of the peptide with the corrin cannot be excluded. The cyclic peptides **4** and **11** showed affinities to aquocobalamin comparable to the natural tripeptide glutathione and linear controls, and lower than values previously reported for dendritic ligands of B_{12}, which are 2- to 5-fold higher than for glutathione

Table 4 Binding affinities for the cyclodecapeptides and linear analogs to aquocobalamin, determined by UV-titration

Peptide	Structure	$K_a/10^5$ [M^{-1}]
3a	c-(HDEPGIATPq)	5.8 ± 1.7
3b	c-(HITPGEDAPq)	6.5 ±.1.2
4	c-(VDEPGEDCPq)	13 ± 5.1
4′	$VDEPGEDCPGNH_2$	16 ± 5.7
4′Ac	$AcVDEPGEDCPGNH_2$	13 ± 8.0
9a	c-(HCTPGELAPq)	32 ± 9.5

(Table 4).[5,13] Linear controls showed comparable binding affinities to the cyclic peptides, which possibly reflects the dominant effect of the acidic amino acids and the cysteine residues present on linear and cyclic peptides. Additionally, the β-turn inducing diad Pro-Gly may induce some preorganization in the linear analogs. Peptide **9a**, with both His and Cys, shows coordination with the cysteine thiol group as expected by the higher affinity of thiol (thiolate) for Co(III) when compared to imidazole (Fig. 2B).[14]

Structure determination of the cobalamin–cyclodecapeptide 4 complex (Cbl–4)

As already observed in peptide dendrimers, amino acid sequence discrimination was also found in the cyclic peptides, with glutamate and aspartate residues prevailing in the hits (Table 2). We postulate that the acidic residues could contribute to binding by interactions with the peripheral side chains of corrin. Such interactions could pre-organize the metal cofactor and peptide or contribute to the binding affinity. In order to investigate the occurrence of peripheral binding interactions, the structure of the **Cbl–4** complex was analyzed by NMR-spectroscopy.

The amino acid signals of the cyclic peptide were assigned by sequential attribution. The chemical shifts of the two cysteine Hβ protons in the B_{12} complex (1.50 and 0.46 ppm, Fig. 3) show lower shielding when compared to the free cyclodecapeptide (3.28 and 2.96 ppm), supporting cobalt coordination at that residue. The conformation of the cysteine side chain was determined by distance constraints for Cys3-H_β/Cys3-H_α and Cys3-H_β/Cys3-NH. Furthermore, five distance constraints were unambiguously determined between the peptide and the cobalamin. Interestingly,

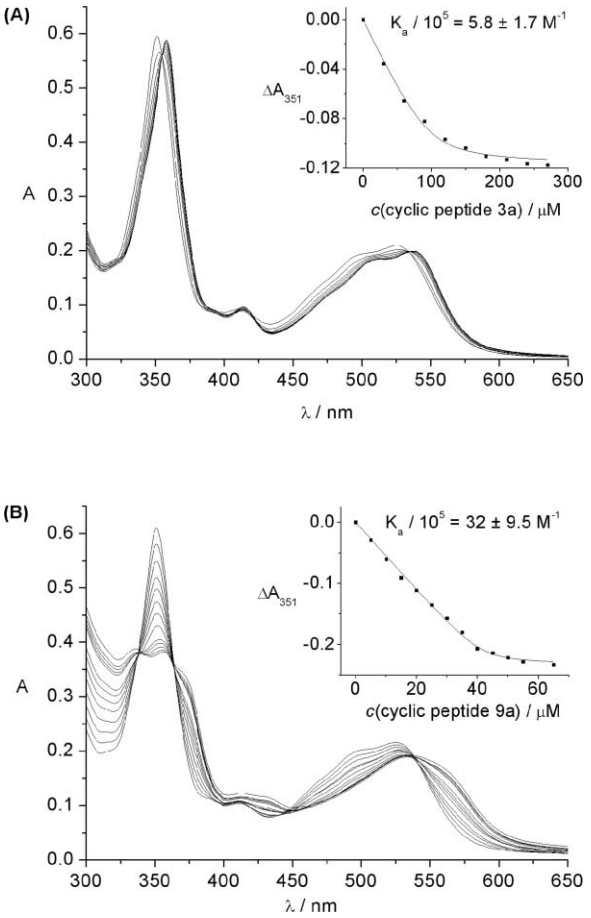


Fig. 2 Representative UV-titration for binding of cyclodecapeptides to aquocobalamin: (A) histidine-cyclodecapeptide **3a**;(B) histidine and cysteine-cyclodecapeptide **9a**.

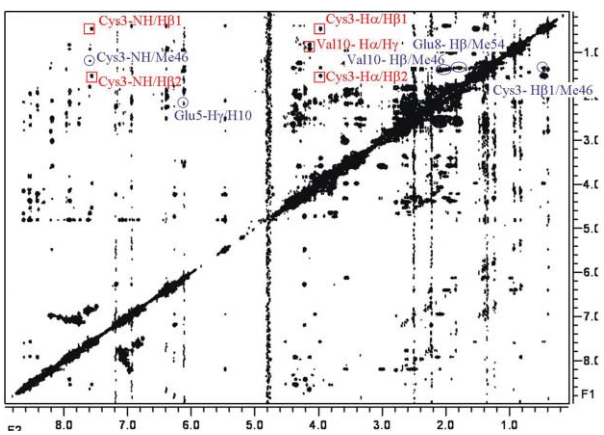


Fig. 3 NOESY-NMR spectrum of the **Cbl–4** complex in H_2O–D_2O (90 : 10). Intramolecular NOEs are in red and intermolecular NOEs are in blue.

the intermolecular NOEs involve the side chains of the three amino acids occurring in higher percentages in the 18 sequences in Table 1; Glu8 is present in 10 hits, Glu5 in 15 and Val10 in 13 sequences. Minimization under these constraints gave a stable model with no distance violations larger than 0.1 Å (Fig. 4).

In the ^{1}H NMR-model obtained, the cyclodecapeptide shields the upper corrin face almost completely, and H-bonding contacts

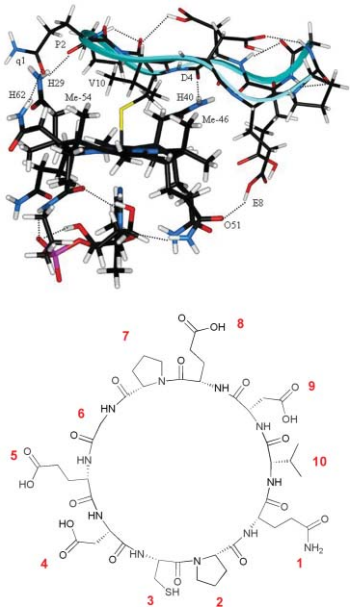


Fig. 4 Structural model for the cobalamin–cyclodecapeptide **4** complex (**Cbl–4**) and the structure of cyclic peptide **4**. Dashed lines represent H-bonds.

are established between the corrin carboxamide side chains and several residues of the cyclodecapeptide. Intermolecular H-bonds involve the carbonyl groups of D-glutamine q1, aspartate D4, and proline P2, with H62, H40 and H29 of cobalamin, respectively. An additional H-bond between the carboxyl group of glutamate E8 and O51 is also observed because the minimization was performed with protonated side chain carboxylic acids. Van der Waals contacts are furthermore predicted between the methyl group of valine V10 and the methyl groups Me-54 and Me-46 of cobalamin. The intermolecular interactions with acidic amino acids correlate with the high frequency of glutamate residues in the hits. It is also interesting that in the X-ray structure of the transport protein Cbl–transcobalamin complex, hydrogen-bonding interactions with Gln and Asp transcobalamin side chains and corrin carboxamide groups are also observed.[12] The large number of additional direct and solvent-mediated contacts in the transcobalamin–Cbl complex probably explains the much higher affinity of the protein to vitamin B_{12} compared with the cyclodecapeptides, also taking into account the entropically favourable dissolvation of B_{12} in the protein complex, an effect which is much more limited in the B_{12}–cyclodecapeptide complex formation due to the limited surface interaction.

Cyanide exchange kinetics

The stability of the B_{12}-cyclodecapeptide complexes was evaluated more closely in the case of cyclic peptide **4** and its histidine analog **4His** by determining the rate of ligand exchange against cyanide, which is reduced in the presence of macromolecular coordination such as by B_{12}-transport proteins.[15] The cysteine coordinating peptide **4** and analogs showed exchange rates comparable to the natural tripeptide glutathione, known to bind strongly to cobalamins (data in the ESI†). In the case of the natural proteins the cobalt center is coordinated to a histidine side chain and therefore **4His** is a better analog to the natural system.

The histidine cyclodecapetide **4His** reduced the rate of ligand exchange by more than 10 fold (Fig. 5), showing that a significant stabilization of the complex was achieved compared to imidazole, suggesting significant interactions of the cyclodecapeptide with the corrin peripheral groups besides the coordinating residue, in agreement with the solution structure (Table 5). Exchanges with linear analogs, with free or acetylated N-termini, were 6 times slower than imidazole.

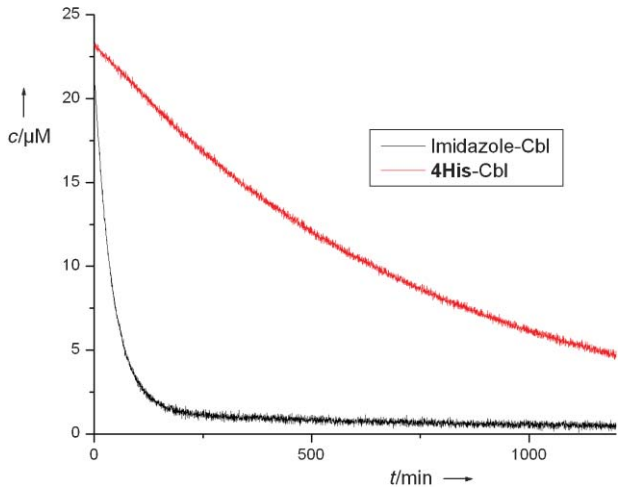


Fig. 5 Conversion of imidazole–Cbl and **4His**–Cbl to cyanocobalamin. Absorption was measured at 560 nm at pH 7.5, 25 °C. Initial rates v/μM min^{-1}: 0.34 for the conversion of imidazole–Cbl and 0.02 for **4His**–Cbl. Both curves were measured until complete displacement of the upper ligand by cyanide.

Conclusion

The experiments above describe the first complex between a vitamin B_{12} derivative and a cyclic peptide. The discovery of this cyclic peptide was made possible by the availability of an efficient combinatorial chemistry protocol for library synthesis, including the on-bead formation of cyclic peptide, a selective binding assay, and a powerful bead-decoding protocol enabled by the TAGSFREE algorithm for library encoding. The key binding interaction occurs by coordination of a nucleophilic residue such as cysteine or histidine to cobalt. The prevalence of Cys containing cyclic peptides as ligands for aquocobalamin could have a thermodynamic and a kinetic origin. Thiolates bind to Co(III) with higher affinity than imidazoles. It is also known that the rate of complexation of nitrogen containing ligands to Co(III) is lower than that of other ligands. In the case of the on-bead screening, the kinetic factor may be predominant since the time of incubation is not very long. Therefore, more cysteine containing cyclopeptides were found in the screening assay, although thiolates and imidazoles are both known ligands for Co(III).[14]

Table 5 Cyanide exchange rates with His-peptides in cobalamin complexes

Ligand in Cbl complex		$t_{1/2}$ for 50% ligand exchange[a]
4His	c-(VDEPGEDHPq)	7–8.5 h (87 μM)
4His′	VDEPGEDHPGNH$_2$	2 h 46 min (87 μM)
4His′Ac	AcVDEPGEDHPGNH$_2$	3 h (87 μM)
Imidazole		30 min (87 μM)
H_2O[b]		<1 s (2.5 mM)
TC[b]	Transcobalamin	50 min (1.0 mM)
IF[c]	Intrinsic factor	20 s (0.5 mM)
HC[c]	Haptocorrin	1 min (0.1 mM)

[a] Half-life $t_{1/2}$ of the ligand–B_{12} complex in the presence of the indicated cyanide concentration (in parentheses). *Conditions*: [Cbl] = 25 μM; 37 °C, pH 7.5 for proteins; 25 °C, pH 7.0–7.5 for other ligands. [b] data from ref 14a; [c] data from ref 14b.

The cyclodecapeptide–Cbl complexes show stability comparable to the tripeptide glutathione–B_{12} complex. Glutamate residues were present in the sequences obtained by on-bead screening, an observation already made with peptide dendrimers that gave hits with polyglutamate shells.[5] However a slower exchange rate of the water axial ligand on the beads was observed with the cyclic peptides, with 2–3 h incubation time required. In comparison, the peptide dendrimers gave Cys containing hits after 15 min. Further studies of the exchange kinetics in the two systems are being performed. The ^{1}H NMR-structure of the complex revealed hydrogen-bonding interactions with the peripheral carboxamides on cobalamin and residues on the peptide ligand, besides metal coordination. The cyclodecapeptide–cobalamin complex **4His**–Cbl displays enhanced kinetic stability against ligand exchange by cyanide when compared to linear analogs and imidazole.

To date only very few model systems of B_{12}-binding proteins have been reported in the literature. These include antibodies[16] and dendritic peptide ligands.[5] Cyclodecapeptide ligands reproduce essential features of previous model systems, yet display a much simpler structure and are particularly easy to synthesize. Screening combinatorial libraries of cyclic peptides with both coordinating residues (Cys and His) enabled the discovery of the first cyclopeptide ligands incorporating either Cys or His. Further investigations on focused libraries with only histidine as the binding residue might give new models for the metal–peptide interaction present in the transport protein transcobalamin. The cysteine containing cyclic peptides however are probably better candidates as adjuvants for vitamin B_{12} due to their faster and stronger complexation to cobalt ions. Further studies with both systems are now in progress.

Experimental

General methods

Protected amino acids and Tentagel Macrobeads® resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France), and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). RP-HPLC was performed on Waters equipment with a 600 controller and a Waters 2487 dual absorbance detector. The peptide derivatives were analyzed on an analytical column (Macherey-Nagel Nucleosil 120 Å 3 μm C_{18} particles, 30 × 4.6 mm, flow rate of 1.3 mL min^{-1} for 15 minutes gradient, Nucleosil 100 Å 5 μm C_{18} particles, 250 × 4.6 mm, flow rate of 1 mL min^{-1} for 30 minutes gradient) using a linear gradient and the following solvent system: water containing 0.1% TFA (solvent A); acetonitrile containing 0.1% TFA and 9.9% H_2O (solvent B). UV

absorbance was monitored at 214 nm and 250 nm simultaneously. Semi-preparative column chromatography (Delta-Pak™ 100 Å 15 μm C_{18} particles, 200 × 2.5 mm) was used to purify crude peptides by using an identical solvent system at a flow rate of 22 mL min^{-1}. The electron spray ionization mass spectrometry (+ESI-MS) was recorded on a VG Platform II (Micromass). The analysis was performed in the positive mode for peptide derivatives using 50% aqueous acetonitrile as the eluent.

Library synthesis

The on-bead cyclic-decapeptides library was synthesized on Tentagel Macrobeads® resin (1.0 g, loading: 0.3 mmol g^{-1}) using the split-and-mix procedure. The resin was acylated with 3 eq. of *N*-α-Fmoc amino acid in the presence of PyBOP (312 mg, 0.6 mmol, 3 eq.) and diisopropylethylamine (DIEA) (260 μL, 1.5 mmol, 6 eq.) in DMF. After 2 × 60 min the resin was washed (3 × 5 mL each) with DMF, CH_2Cl_2, and MeOH and controlled with the Kaiser and TNBS (trinitrobenzenesulfonic acid) tests followed by acetylation. The Fmoc protecting groups were removed by treatment with piperidine, 20% in DMF (3 mL, 3 × 10 min), and then washed with DMF, CH_2Cl_2, and MeOH. Split and mix was carried out after α-amine deprotection. The resin was suspended in DMF : CH_2Cl_2 (2 : 1, v/v), mixed *via* nitrogen bubbling for 15 min and distributed in five equal portions for the next coupling. After the last coupling step, the Fmoc protecting group was removed. The resin bearing the library of decapeptides (loading: 0.21 mmol g^{-1}, quantified by measuring the absorption of fluorenylmethylpiperidine adduct at 299 nm) was dried under vacuum for 1 h and swollen in a glass reactor fitted with a sintered glass frit with dry CH_2Cl_2 (10 mL, 2 × 15 min) and dry DMF (10 mL, 1 × 15 min). The resin was treated with $PhSiH_3$ (2.6 mL, 21 mmol) in dry CH_2Cl_2 (10 mL) for 5 min; $Pd(PPh_3)_4$ (61 mg, 0.052 mmol) was then added and the resin was stirred under argon for 20 min. The reagents were removed by filtration, the resin washed with CH_2Cl_2 (10 mL, 4 × 1 min) and DMF (6 mL, 3 × 1 min) and the procedure was repeated once. The resin was finally washed with CH_2Cl_2 (10 mL, 2 × 1 min), dioxane–water (9 : 1, 10 mL, 2 × 1 min), DIEA 5% in DMF (6 mL, 3 × 1 min) and DMF (10 mL, 2 × 1 min). On-bead cyclization was performed following the acylation procedure described above. The protecting groups were removed on-bead by treatment with a TFA–H_2O–iPr$_3$SiH–1,2-ethanedithiol (94 : 2.5 : 1 : 2.5, v/v) solution for 2–3 h. This procedure was repeated once. The reagents were removed by filtration and the resin washed with CH_2Cl_2 (10 mL, 5 × 1 min), MeOH (10 mL, 2 × 1 min) and CH_2Cl_2 (10 mL, 5 × 1 min).

On-bead B_{12}-binding assay

10 mg of dry resin containing the cyclodecapeptide library was kept in PBS buffer (20 mM, 150 mM NaCl, pH 7.4) for swelling for 30 minutes. The buffer was then removed and 4 mL of aquocobalamin solution (100 μM) in PBS buffer were added. After stirring for 2.5 hours, the aquocobalamin solution was removed by filtration and the resin was washed six times with PBS buffer. The resin was suspended in PBS, spread on a silica plate and the most intensively red colored beads were picked for sequence determination.

Bead analysis and decoding

Single-peptide-containing resin beads were hydrolyzed with aqueous HCl (6 M) at 110 °C for 22 h, and their amino acid composition was determined quantitatively by HPLC after derivatization with phenyl isothiocyanate (PITC). The sequences were then assigned by TAGSFREE decoding.[10]

Decapeptide SPPS

The on-bead decapeptides were synthesized on Rink Amide MBHA® resin (0.1 mmol scale) with an automated synthesizer (433A from Applied Biosystem) using Fmoc/*t*Bu chemistry. 10 eq. excess of protected amino acids, HBTU coupling reagent and 20 eq. excess of DIEA were used. The FastMoc 0.1 mmol program (SynthAssist™ version 3.1) was used with a single coupling. Cyclic peptides **3a**, **4**, and **4His** were cyclized on-bead after Fmoc and allyl deprotection as described above in the library synthesis section. The resin was treated for 2–3 h with TFA–H_2O–iPr$_3$SiH (95 : 2.5 : 2.5, v/v) for **3a** and **4His** or TFA–H_2O–iPr$_3$SiH–1,2-ethanedithiol (94 : 2.5 : 1 : 2.5, v/v) for **4**. After evaporation and precipitation in diethyl ether, the cyclic peptides were purified by semi-preparative RP-HPLC. Peptide **3a**: 41 mg (79%); t_R = 5.6 min (5–100% B in 15 min); +ESI-MS: calcd for $C_{45}H_{68}N_{13}O_{16}$ 1046.5, found: m/z 1046.6 $[M + H]^+$. Peptide **4**: 46 mg (86%); t_R = 7.9 min (5–40% B in 15 min); +ESI-MS: calcd for $C_{43}H_{64}N_{11}O_{19}S$ 1070.4, found: m/z 1070.6 $[M + H]^+$. Peptide **4His**: 32 mg (58%); t_R = 6.6 min (5–40% B in 15 min); +ESI-MS: calcd for $C_{46}H_{66}N_{13}O_{19}$ 1104.5, found: m/z 1104.6 $[M + H]^+$. Linear peptides **4′** and **4His′** were cleaved by treatment of half of the resin with TFA–H_2O–iPr$_3$SiH–1,2-ethanedithiol (94 : 2.5 : 1 : 2.5, v/v) or TFA–H_2O–iPr$_3$SiH (95 : 2.5 : 2.5, v/v) respectively. Linear acetylated peptides **4′Ac** and **4His′Ac** were obtained by treatment of the second half of resin with Ac_2O–pyridine–DMF (1 : 2 : 7) solution before cleavage. After evaporation and precipitation in diethyl ether, the linear peptides were purified by semi-preparative RP-HPLC. Peptide **4′**: 46 mg (82%); t_R = 5.0 min (5–100% B in 15 min); +ESI-MS: calcd for $C_{40}H_{62}N_{11}O_{18}S$ 1016.4, found: m/z 1016.4 $[M + H]^+$. Peptide **4′Ac**: 40 mg (76%); t_R = 5.7 min (5–100% B in 15 min); +ESI-MS: calcd for $C_{42}H_{64}N_{11}O_{19}S$ 1058.4, found: m/z 1058.4 $[M + H]^+$. Peptide **4His′**: 35 mg (66%); t_R = 4.5 min (5–100% B in 15 min); +ESI-MS: calcd for $C_{44}H_{66}N_{12}O_{18}$ 1050.5, found: m/z 1050.4 $[M + H]^+$. Peptide **4His′Ac**: 38 mg (69%); t_R = 6.0 min (5–100% B in 15 min); +ESI-MS: calcd for $C_{45}H_{66}N_{13}O_{19}$ 1092.5, found: m/z 1092.5 $[M + H]^+$. HPLC and +ESI-MS data for the other peptides prepared and isolated using the same procedure are found in the ESI.†

UV–vis titrations

1000 μL of a *ca.* 20 μM buffered solution (exact concentration was determined by measuring absorption: ε_{351} = 25 000 $dm^3\ mol^{-1}cm^{-1}$ in 20 mM HEPES buffer, pH 7.0) of aquocobalamin was prepared in a gas tight cuvette. The titration was carried out by adding portions of a stock solution of peptide (1.25 mM) with a gas tight syringe and waiting until no further change in the spectrum took place. Data points were collected at 351 nm for Cys peptides (equilibration time 1.5–3 h) and at 358 nm for the UV–vis titrations of His containing peptides (4 h equilibration time) and fitted using a single site binding equation.[17]

Cyanide exchange kinetics

The peptide complexes were formed prior to the exchange measurement by dissolving the peptide (10 mM) in a solution of aquocobalamin (1 mM in HEPES buffer pH 7.5). The imidazole complexes were formed in the same way. All exchange experiments were performed at 25 °C and in parallel with an imidazole measurement for direct rate comparison.

For the exchange measurement HEPES buffer (975 μL, 20 mM, pH 7.5) containing 87 μM NaCN was mixed with peptide complex solution (25 μM) in a cuvette. The concentration of aquocobalamin, determined by measuring the absorption at λ_{max}/nm: 352, served as a basis for the starting concentrations of the complexes.

After the addition the conversion of the cobalamin complexes to cyanocobalamin was detected by measuring the absorption at 560 nm over time. Under these conditions, according to the observed absorbance spectrum, only cyanocobalamin (λ_{max}/nm: 361, 550, 522) is formed.

The initial rates of cyanocobalamin formation were obtained by linear regression using the first 20 data points. The $t_{1/2}$ of cyanocobalamin formation was determined by calculating the mean value between the absorbance at $t = 0$ and the absorbance at cyanocobalamin saturation and reading the corresponding time.

Cyanide exchange kinetics of the Cys containing peptides were performed in the same way with the following changes: concentration of Cys-peptide: 2.5 mM; HEPES buffer pH 7.0; concentration of CN^-: 81 μM; every exchange experiment was done in parallel with a glutathione measurement for direct rate comparison; absorption was measured at 361 nm under oxygen-free conditions.

NMR-spectroscopy and modeling

NMR spectra were obtained at 500 MHz with a Varian Unity Plus spectrometer. Samples were dissolved in a mixture of 95% H_2O–5% ^2H_2O or 99.9% ^2H_2O to a final concentration of 1 mM at 25 °C. A set of two-dimensional (2D) spectra, including TOCSY,[18] DQF-COSY,[19] ROESY[20] and NOESY[21] were acquired with 2 s steady state recovery times, mixing times (t_m) of 60 ms for TOCSY and 250 ms for NOESY. Water suppression was achieved by appending an excitation-sculpting module[22] to the non-selective detection pulse and with selective Gaussian-shaped pulses of 3–5 ms. Spectra were acquired in the phase sensitive mode using TPPI,[23] or States method.[24] The spin-lock mixing of the TOCSY experiment was obtained with DIPSI-2 pulse trains at $\gamma B_2/2\pi = 9$–10 kHz.[25] The acquisitions were performed over a spectral width of 10 ppm in both dimensions, with matrix size of 1024–2048 data points in t_2 and 256–512 points in t_1, and 32–128 scans/t_1. All spectra were referenced with external TSP-d_4. Data processing and analysis were performed using Felix software (version 2001, Accelrys, San Diego, CA, USA) with shifted (60–90 degrees) square sinebell apodization and polynomial baseline correction for NOESY data.

Approximate interproton distance restraints were calculated using the isolated two-spin approximation relationship, $r_{ij} = r_{kl}\ (\sigma_{kl}/\sigma_{ij})^{1/6}$, where σ_{ij} and σ_{kl} are the NOE intensities for the atom pairs i, j and k, l separated by distances r_{ij} and r_{kl}, respectively. Cross-peaks from cysteine Hβ were used as calibrants. Stereospecific assignment for the cysteine β-methylene protons were achieved by combining qualitative analysis of $^3J_{H\alpha-H\beta}$ coupling constants extracted from a DQF-COSY spectrum, and interproton Hα–Hβ distances determined from ROESY spectrum. Restrained energy minimizations were performed by using the Insight II/Discover software (Version 2005, Accelrys, San Diego, CA, USA), using the set of distance and dihedral restraints determined by NMR. The selected force field was ESFF, and, to shorten the range of Coulomb interaction, a distance-dependent relative dielectric constant, ε_r, was used ($\varepsilon_r = 4r$). The structure was subjected to 2500 iterations of steepest descent minimization, followed by 2500 iterations of conjugate gradient minimization and the convergence of minimization was followed until the RMS derivative was less than 0.01 kcal mol^{-1}.

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