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Conformational Studies of Macrocyclic Corrin-Ring of Coenzyme B_{12} by NMR methods

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Abstract: An enzyme derived conformational changes of cobalamine is thought to be important in the homolytic cleavage of Co–C bond which is the first step of catalytic C1-cycle of coenzyme B_{12}-dependent enzymes. Modern 2D-NMR and NMR-based distance geometric studies were carried out to determine the 3D structure of corrin ring. Homonuclear and heteronuclear correlation NMR experiments were performed for complete ^{1}H-NMR signal assignments. Distances between numerous proton pairs were deduced based on the NOE cross peak intensities and subsequently used as input into the distance geometry program for the 3D structure determination. The detailed 3D structure from the present NMR-based analysis was compared with the result from X-ray crystallographic study, which revealed greater conformational changes occur in benzimidazole group and sugar ring than in macrocyclic corrin and tetrapyrrole. In addition, the distance geometry used in this study was found to be quite useful for NMR-based structure determination of medium-sized molecules that give poor NOE effects arising from their intermediate tumbling rate ($\omega\tau_c \approx 1.0$).

INTRODUCTION

An enzyme derived conformational changes of cobalamine is thought to be important in the homolytic cleavage of Co-C bond which is the first step of catalytic C1-cycle of coenzyme B_{12}-dependent enzymes.[1] Since the importance of B_{12}-dependent catalysis, cobalamins have been received great attention for last two decades. NMR studies have been also widely made to accomplish complete ^{1}H- and ^{13}C-NMR signal assignments and structural features enhancing the knowledge of C1-cycle mechanism.[2-3] B_{12} has a

ing cobalt at the center, and Co(II)/Co(III) oxidoreduction associated with the conformational changes are believed to be important in catalysis. Macrocyclic corrin originates from the 8x δ-aminoleuvulinc acid *via* uroporphyrinogen III and sirohydrochlorin.[4] Molecular structure with numbering scheme are shown in Fig 1. Resonance signal assignments were made mostly based on the B_{12} model compounds, ^{13}C relaxation, analyses of paramagnetic line broadening, pH-dependent studies, and heteronuclear coupling constant analysis. Modern 2D-NMR techniques including homonuclear and heteronuclear correlation experiments and distance geometry have been utilized to elucidate the solution-state structure.[5-6] Dynamic structural features of four flexible tetrapyrrole side chains and a benzimidazole axial ligand give rise to poor NOE observations at ambient temperature. NOESY experiments with various mixing time and spin-lock NMR experiments have been carried out to overcome the poor NOE observation.[3]

EXPERIMENTAL

Preparation of Sample

Reagents of cyanocobalamine (Sigma) and hydroxocobalamine acetate (Roussel) were purchased and used without further purification. NMR samples of coenzyme B_{12} derivatives including cyano-cobalamine and hydroxy-cobalamine (adjusted to pH 8.5 with NaOD) were prepared by once lyophilizing and then dissolving in 500 μL of D_2O.

NMR Data

^{1}H-NMR spectra were recorded on Varian Unity-500 MHz and Mercury 300 MHz spectrometer. Spectra were typically recorded using the samples dissolved in 99.98% D_2O with the following conditions: 90° pulses presaturation of HOD; 16K data points; 0.5 Hz line broadening; and accumulation of 32 scans. As internal references for ^{1}H- and ^{13}C-NMR signal assignment HOD and 3-(triethylsilyl) propionate were used, respectively. Raw NMR data were transferred via ethernet to SGI INDY and IBM 43P workstations, converted into readable format, and processed with programs HYNMR. HOHAHA, COSY, Phase-Sensitive NOE, HMQC, HMBC spectra were obtained with a 512 x 2048 data matrix size and 16 scans per t1 and were zero-filled with 2048 x 2048 real points. The delay time between successive scans and the mixing time were, respectively, set 1.2 s and 300 ms. Gaussian line broadening of 2 Hz was used in the t2 dimension before Fourier transformation. A 90° shifted sinebell squared filtering was used in the t1 dimension before Fourier transformation. For NMR-based DG computations and identification of spin diffusion peaks NOESY data were collected at five different mixing times of 50, 100, 200, 300, 500 ms.

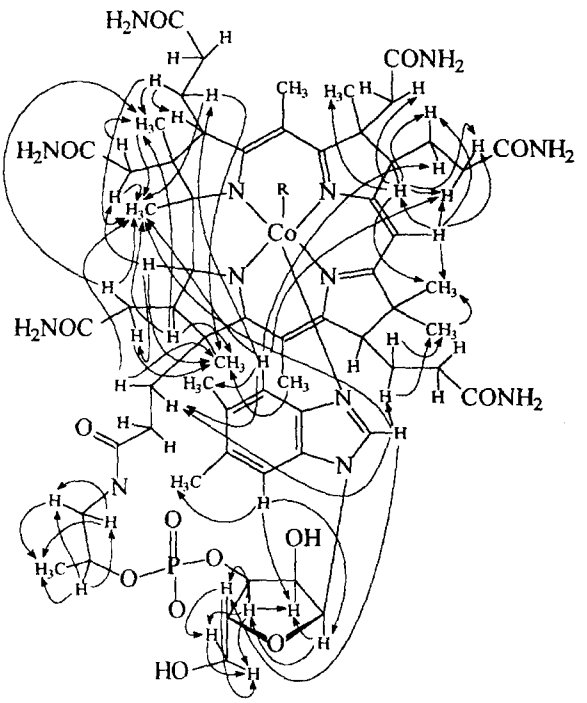

Fig. 1. Important through-space connectivities of cyano-cobalamine with numbering scheme where R is CN.

Structure Determination

The NOE restraints were obtained via qualitative assessment of NOE cross peak volumes in the NOESY spectra. However, no restraints were included to attempt to fit the spin diffusion cross peaks. Instead, loose NOE restraints were used for cross-peaks classified as strong, medium, weak, and very weak. The ranges of initial NOE restraints were assigned with 2.0-2.75 A(strong cross peak intensities), 2.0-3.5 A(medium cross peak intensities), 2.0-4.5 A(weak cross peak intensities), and 3.5-5.0 A(very weak peak intensities).

Distance geometry (DG) structures were generated and refined by using primary restraints and loose NOE-derived distance restraints. Trial distances generated by selecting random distances between the upper and lower bounds of each element were embedded in 3-dimensional space by the metric matrix method and subjected to simulated annealing (SA) and conjugated gradient minimization (CGM). The embedded initial coordinates containing violations of the upper and lower boundary restraints were then refined. After minimization to a moderate target penalty (*ca.* 0.3 A^2), the initial DG structure was saved and new DG structures were generated by performing two 10 A randomizations of atom positions, followed by SA and CGM refinement. Once a low-penalty (penalty = squared sum of the covalent and experimental bounds violation) structure was stored, new starting coordinates were then obtained by performing different embedding, SA, CGM algorithms. Further refinement was achieved by application of variable velocity simulated annealing (to maximum penalty values of 10-20 A^2), SHAKE (to penalties of ca.10 A^2) and CGM algorithms.

The complete time course for nuclear relaxation was then determined for each refined structure *via* numerical integration of the Bloch equations. As described previously, this approach accurately accounts for spin diffusion. Generic Z-leakage rate constant *Kzl*, accounting for the loss of Z-magnetization within mixing period, was taken to be 1 s^{-1} (3 s^{-1} for Me20, Me25, Me35, Me46, Me47, Me53, Me54, Pr3, B10, B11). The cross relaxation rate constant(*Kcr*) which governs the cross relaxation rate was set to be 63 sec in this NOE back-calculation which is very close to that used for coenzyme F430.[4-5] *Kcr* was determined by using NOE build-up curve comparisons in advance with well resolved geminal protons which is structurally known to have internuclear distance of 1.8 A. The cross relaxation rate term includes intramolecular dipolar relaxation, effect of chemical exchange, quadrapolar relaxation, the possible contribution from spin rotation, scalar relaxation, and multiple quantum effects. The 2D NOE back-calculation gives a list of normalized auto- and cross peak intensities for selected mixing times in increasing order. Profiles of these outputs provide the theoretical NOE build-up curves. The results of back-calculation are then assigned to GENNOE in order to generate the theoretical 2D NOEs. A consecutive serial file, obtained from GENNOE calculation for different mixing times, are incorporated into HYNMR to generate 2D NOE back-calculated spectra which can be directly compared with experimental spectra. The back-calculated 2D NOE spectra of a DG-NOE structure (lowest penalty value, 0.12 A^2) were found to be consistent with experimental NOE spectra.

RESULT AND DISCUSSION

Complete ^{1}H- and ^{13}C-NMR signal assignments were made by comparing our results with previous papers published for 5'-deoxyadenosylcobalamine.[3] NOE cross peak intensities were classified into four different groups for the DG computations. The NOE

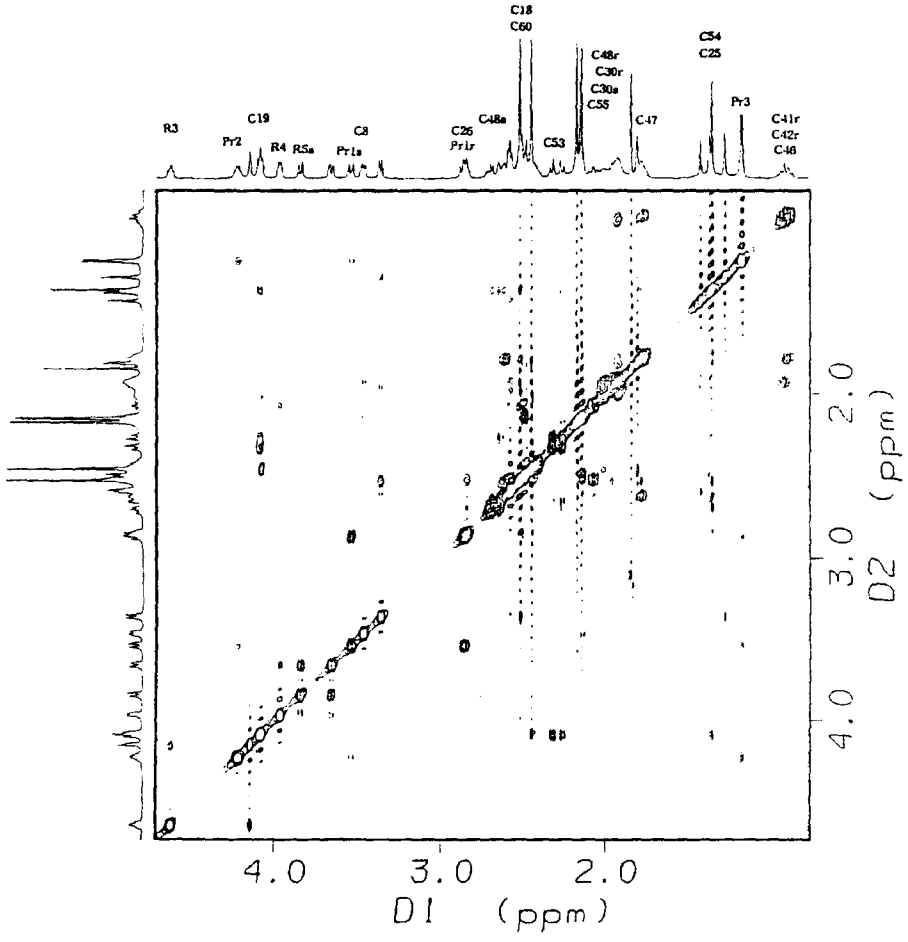

Fig. 2. A portion of 2D NOESY spectrum recorded with 300 ms mixing time.

spectra measured at five different mixing times of 50, 100, 200, 300, 500 ms enabled us to identify the relay peaks arising from the spin diffusion effect that might not be used for structure determination. A portion of 2D NOESY spectrum recorded at 300 ms mixing time is shown in Fig. 2. In addition, some important NOE connectivities near cobalt-containing corrin ring that are used for the structure determination are also shown in Table 1 and in Fig. 1. The results show that much of conformational changes occurring in benzimidazole group and sugar ring are greater than those occurring in macrocyclic corrin and tetrapyrrole. Superposition of X-ray structure on DG structures exhibits a good structural consistence for the entire corrin macrocyclic skeleton(Fig.3).

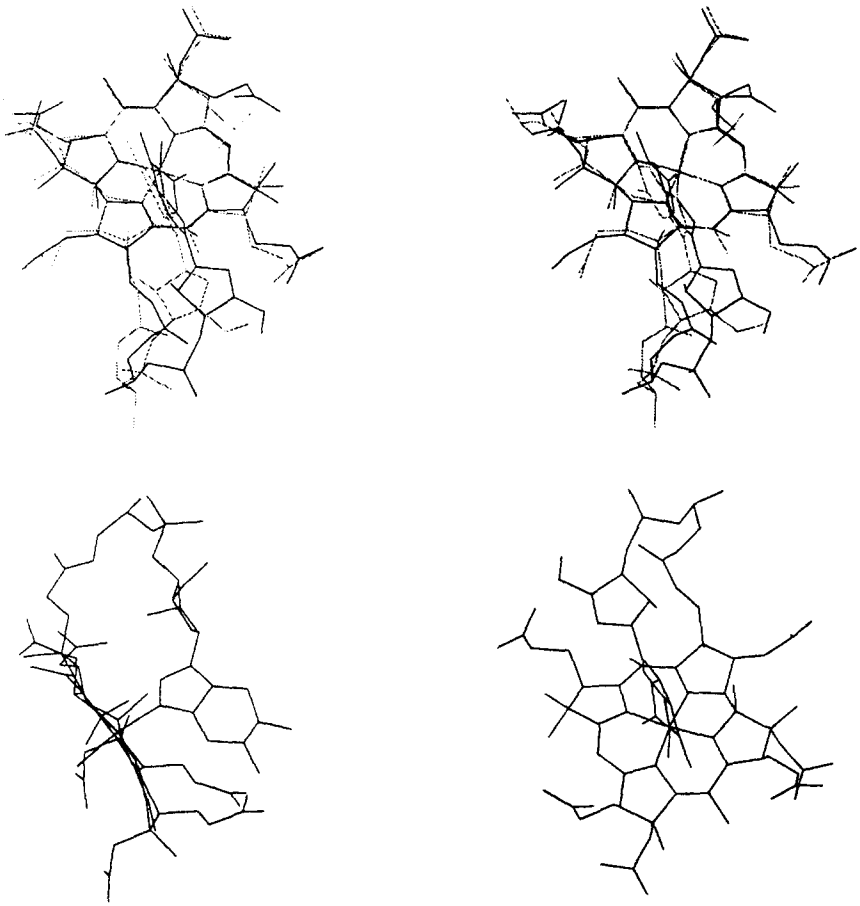

Fig. 3. NMR-based solution state structure with different 90 degree view (up) and NMR structure superimposed with the X-ray crystal structure for the cyano-cobalamine.

The current method of solution-state structure determination for medium-sized molecule is an extended version of the NMR method used for structure determination of large biomolecules and was found suitable for our purpose here. The distance geometry employing spin-lock NOESY was quite useful for NMR-based structure determination of medium sized molecules for which the condition $\omega\tau_c = 1.0$ results in poor NOE effects. Although DG-NOE structures have low penalty value (lowest penalty value, 0.12 A^2), for better refined structure the NOE build-up comparison for each proton pair in the DG structure determination procedure may be necessary.

Table 1. Summary of important NOE connectivities used for the structure determination.

H-Signals	Chemical Shift(ppm)	NOE –Connectivities	HOHAHA-Connectivities
B7	7.14	B11(vw),R2(vw),R1(w)	
B2	6.81	C48r(vw), R4(vw), C20(vw) C55r(w)	
B4	6.39	C41r(vw), C20(vw), C42r(vw), C55r(vw), C30r(vw), B10(w) B11(vw)	
C10	6.14	C46(vw), C42s(vw) C41s(vw), C42r(vw), C8(w)	
R1	6.22	R2(s), R3(s)	
R3	4.64	R5r(m), R5s(m), R4(m), R2(s)	R2, R4, R5r, R5s
Pr2	4.24	Pr3(m), Pr1r(m), Pr1s(m)	Pr1r, Pr1s
C19	4.12	C54(vw), C26r(vw), C60r(m)	C18, C60
R4	3.99	R5r(m), R5s(m)	R2, R5r, R5s
R5s	3.82	R5r(s)	R5r
Pr1r	3.56	Pr3(w), Pr1s(s)	Pr1s, Pr3
C8	3.49	C46(vw), C42r(vw), C42s(vw), C41s(vw), C36(vw), C37r(m)	C42
Pr1s	2.88	Pr3(w), B2(w)	Pr3
C26	2.88	C20(vw), C25(m)	
C60	2.68	C20(vw), C25(vw), C54(m)	C18
C18	2.68	C20(vw), C25(m), C54(w)	
C53	2.32	C54(vw)	
C48s	2.08	C47(w)	
C30s	2.02	C3(w), C25(vw), C20(vw)	
C48r	1.96	C47(m)	
C30r	1.94	C54(w), C20(vw)	
C55	1.94	C54(w), C20(vw)	
C47	1.80	C46(w)	
C25	1.39	C20(m)	

s, m, w ,vw refer to strong, medium, weak and very weak NOE cross-peak intensities respectively.

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