A Multinuclear Solid-State NMR Analysis of Vitamin B$_{12}$ in Its Different Polymorphic Forms

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Abstract: Multinuclear solid-state nuclear magnetic resonance methods (^{59}Co, ^{13}C, ^{15}N, and ^{31}P NMR) were applied at natural abundance to the structural and dynamic analysis of cyanocobalamin (vitamin B$_{12}$) polymorphs. These studies involved recrystallizing a series of samples under different conditions and from various solvents, and subsequently recording their powder NMR spectra at different temperature. Two polymorphs could be identified in these studies, in correspondence with the two structures described by Hodgkin and co-workers in their seminal vitamin B$_{12}$ crystallographic analyses. Most informative about the molecular differences characterizing these two forms were the ^{13}C NMR data, which showed sharp and well-resolved resonances indicative of high sample crystallinity. Diagnostic differences between these two forms could be observed in the chemical shifts of particular resonances, many of which could be assigned to their molecular sites on the basis of solution-state literature and of solid-state spectral editing procedures. These shifts indicated a conformational variability that involved a few specific sites in the corrinoid ring and which was not entirely evident from differences among the X-ray structures previously reported for the forms. Further evidence about the conformational flexibility of these sites in the solid is furnished by ^{13}C spectral shifts observed upon changing temperatures. The relevance of these observations within the context of the extensive structure/ activity relation studies that have been carried out on this class of systems is briefly addressed.

Introduction

Vitamin B$_{12}$ (Scheme 1) and other naturally occurring cobalamins count among the most intensively studied compounds in chemistry.[1−3] Interest in these large biomolecules is stimulated by the roles that B$_{12}$ coenzymes play as cofactors in a series of essential enzymatic reactions related to nucleic acid, protein, and lipid syntheses.[4−6] Central to these catalytic activities is the rupture of the unusual carbon−cobalt covalent bond present in these molecules, and the close interaction postulated to exist between the homolysis of this bond and the conformation adopted by the cobalt's remaining ligands in the holoenzymatic complex.[5−9] In view of this relationship between molecular conformation and enzymatic activity much attention has been focused on determining the precise structure of the cobalamin complex, and particularly on the corrinoid's geometry satisfying the four in-plane cobalt ligand bonds. The first breakthrough in this realm came with the investigations of Hodgkin and co-workers, who by solving the X-ray diffraction patterns of vitamin B$_{12}$ and of several of its chemical derivatives laid the basis for the numerous subsequent structural investigations in this field.[10−14] These have included structure determina-

Scheme 1. Structure of Vitamin B$_{12}$ and Atomic Numbering Used in This Study

Vitamin B$_{12}$
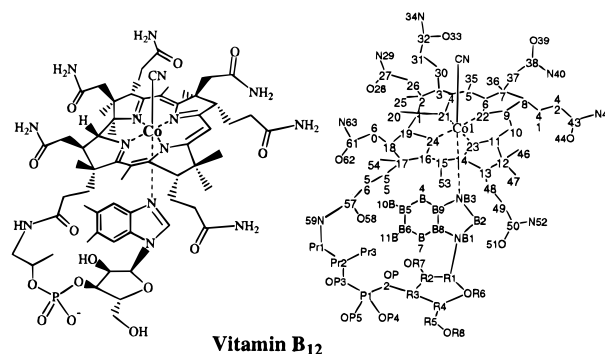
tions of base-on cobalamins and of numerous cobalt-containing model derivatives,[13−18] as well as studies on adenosylcobalamin incorporated into holoenzymatic complexes.[19,20] These diffraction studies, coupled to related chemical and biochemical

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investigations, are at the center of much of what is known about structure/activity relations in this class of compounds. One of the features they suggest is that the kinetics and thermodynamics of the Co−C bond dissociation are strongly modulated by the chemical nature of the opposite axial ligand, as well as by sterical effects arising from "upward" deformations of the corrinoid ring which are made possible by the latter's semi-flexible nature.

Spectroscopic methods of analysis have also been extensively applied to shed light on the relation between cobalamins reactivity and their structural and electronic nature. Particularly important has been the advent of modern pulsed NMR methods, which stimulated investigations on the solution-phase electronic and geometric nature of the molecules as revealed by the ^{13}C chemical shifts of various sites and by specific *J*-coupling constants or Overhauser enhancements between ^{1}H NMR resonances.[17,21−27] Central to the success of these studies was the introduction of multidimensional NMR, which enabled full and unambiguous resonance assignments for these otherwise overtly complex chemical compounds. It has also been recently shown that NMR observations on the ^{59}Co isotope itself, particularly in the solid state, could constitute yet another source of information regarding the electronic structure surrounding the metal.[28,29] Furthermore, such solid-phase measurements could help bridge the gap between the structural information available from diffraction studies on crystals and the combined structure/electronic insight available from solution-state chemical shift or coupling analyses. One of the features noticed in these solid-state NMR investigations was a substantial sensitivity of the cobalt's spectral parameters, particularly of the metal's quadrupole coupling constant, to the physical preparation details of the analyzed powdered samples.[28] The present study expands on this observation by presenting a more extensive investigation on the solid-state NMR spectra displayed by cyanocobalamin powders as a function of their crystallization conditions. In accordance to what was reported in Hodgkin's original series of studies, two polymorphs could be detected for this complex by solid-state NMR. The conditions under which these were obtained were slightly different from those described in this earlier literature, yet they could be clearly differentiated and ascribed in terms of their different ^{59}Co, ^{13}C, and ^{15}N NMR line shapes. One of the most interesting features of these spectra was revealed by carbon NMR, which showed differences that involved few but well-defined positions of the macrocyclic ring and of the axial substituents. This in turn provided an insight about specific conformational differences between the polymorphic forms, which had gone unnoticed from sole analysis of the diffraction data. Also interesting to observe were reversible temperature dependencies on certain particular resonances which are apparently reflecting temperature-dependent

packing forces, and that further stress the flexibility character-izing certain positions within the corrinoid macrocycle. The meaning of these observations as well as some potential future research directions that derive from them are briefly discussed.

Experimental Section

Cyanocobalamin samples were purchased from Sigma and Fluka, recrystallized from a variety of solvents and conditions as specified below, then inserted into suitable containers for the solid-state NMR measurements. These were carried out at magnetic fields strengths of 11.8 and 7.1 T using two similar laboratory-built broadband spectrom-eters: the higher field one was used for the ^{59}Co NMR static acquisitions; the lower field one was used for acquiring ^{13}C, ^{15}N, and ^{31}P magic-angle-spinning (MAS) NMR data. Spectroscopic details about the wide line quadrupolar NMR experiments carried out on cobalt have been presented in detail elsewhere;[30,31] basically, the protocol employed for this spin-7/2 nucleus enabled the acquisition of ideal-like central transition powder line shapes even in the presence of large quadrupolar couplings by the combined use of frequency-swept spin−echo experi-ments, coupled to short (3 μs) and very weak (5 kHz nutation rates) radio frequency pulses. To span an appropriate bandwidth these experiments typically involved the collection of three full-echo data sets (50 μs echo delays) separated by 75 kHz offsets; each of these were acquired with 500 kHz spectral windows and using an average of 10000 scans separated by 100 ms recycle delays. The high-resolution spin-1/2 spectra were collected on a 4 mm doubly tuned NMR probehead using H−X cross-polarization (CP) at 40 kHz, proton decoupling at 100 kHz during the acquisition, and sample spinning with rates at or above 7 kHz. Contact times in these experiments were optimized for achieving the maximum signal to noise (2 ms for ^{13}C and ^{31}P, 9 ms for ^{15}N), and the recycle delays used in all these experiments were 2 s. Occasional use of alternative sequences such as short-CP or dipolar-dephasing, INEPT-based editing, 2D separate local field NMR, or variable spinning speed acquisitions was also made to aid in assigning the ^{13}C spectra, for assessing molecular mobility and measuring shift anisotropies. Variable-temperature operation was achieved in all cases by setting the temperature of a room temperature air or low-temperature nitrogen gas stream to the desired values using laboratory-built controllers based on Omega devices. Chemical shifts were determined using 1 M aqueous $K_3[Co(CN)_6]$, adamantane, $(^{15}NH_4)_2SO_4$, and triphenylphosphine as external references for the ^{59}Co, ^{13}C, ^{15}N, and ^{31}P NMR acquisitions, respectively.

Results and Discussion

^{59}Co NMR Data. Figure 1 compares a series of solid-state ^{59}Co NMR spectra of vitamin B_{12} powders crystallized from various solvents and under different conditions, collected at the same (room) temperature. Despite the limited resolution of these central transition patterns three different line shapes can be clearly distinguished among the spectra: a relatively featureless one stemming from either the sample as purchased or after subjecting an aqueous B_{12} solution to fast vacuum evaporation of the solvent (Figure 1a), a broader pattern arising from an aqueous B_{12} solution that has been left to slowly evaporate at high (60−70 °C) temperatures (Figure 1b), and a narrower pattern that can be observed after crystallizing the cobalamin at room temperature from a wide variety of solvents (Figures 1c−g). The "as-purchased" pattern can actually be closely reproduced by subjecting either of the other two line shapes to a severe line broadening, suggesting that a distribution of coupling parameters is present among the crystallites of this amorphous sample. The other two line shapes on the other hand have relatively sharp singularities characteristic of crystalline

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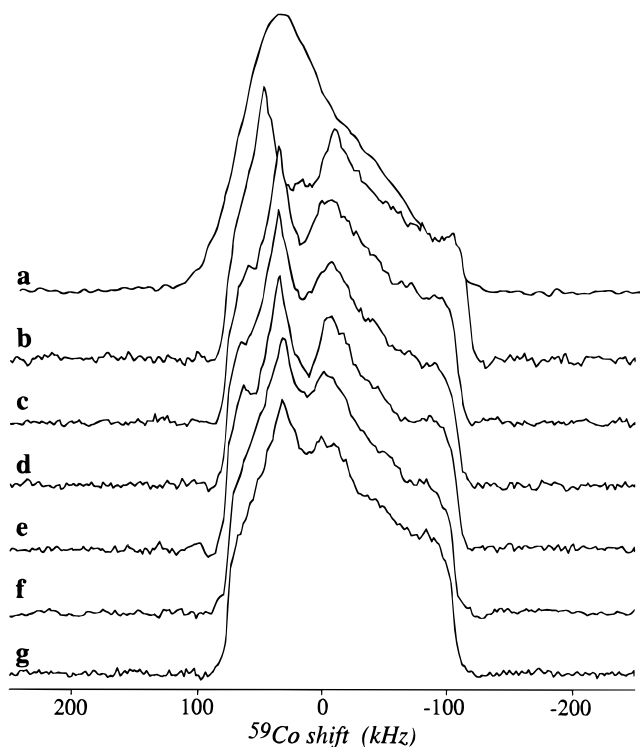
Figure 1. 120 MHz (11.7 T) ^{59}Co NMR line shapes acquired from vitamin B_{12} powders crystallized under the following conditions: (a) the as-purchased sample; (b) slow evaporation of an aqueous solution at 65 °C; (c) room-temperature crystallization from H_2O/acetone; (d) room temperature crystallization from isopropyl alcohol; (e) room-temperature crystallization from ethanol; (f) room-temperature crystallization from methanol; and (g) room-temperature crystallization from H_2O. All powders were prepared from the latter specimen, and all spectral changes could be reproduced upon redissolution and reprecipitation. Spectra were collected on ca. 50 mg of sample using the spin–echo approach detailed in ref 31.

Figure 2. Comparison between static ^{59}Co NMR line shapes acquired from vitamin B_{12} samples obtained under different conditions and their ideal best fit simulations. The latter assumed 2500 different orientations, a 2 kHz Gaussian line broadening, and identical chemical shift tensor parameters: isotropic shift (4650 ± 100) ppm downfield from saturated aqueous $K_3[Co(CN)_6]$, shift anisotropy $\delta_{CS} = (-635 \pm 100)$ ppm, chemical shift asymmetry parameter $\eta_{CS} = 0.2 \pm 0.1$, Euler angles defining the relative orientations between the electric field gradient and chemical shielding tensor $(\alpha, \beta, \gamma) = (45 \pm 20°, 40 \pm 20°, 20 \pm 20°)$. Only the quadrupole coupling constants differed between these two crystalline forms: $e^2qQ/h = 27.8 \pm 0.3$ MHz, $\eta_q = 0.1 \pm 0.1$ for the water ("wet") sample, and 26.1 ± 0.4 and 0.1 ± 0.1 for the ethanol (and remaining "dry") samples. Error intervals in these parameters describe the ranges within which acceptable (visual) agreement with the experimental shapes was observed.

samples; their best fit simulations in terms of the ^{59}Co quadrupolar and shielding tensor parameters are summarized in Figure 2.

It is tempting to correlate the different types of ^{59}Co NMR spectra observed in these two polycrystalline samples with the two crystallographically distinct forms described in Hodgkin's original studies.[10–12] These included a "wet" form, possessing several waters of hydration and stable over long periods only in its mother liquor, and a "dry" form, characterized by a smaller unit cell and which results from the "wet" one when it is removed from the solvent and loses water of cocrystallization. Preliminary experiments carried out toward assigning our NMR samples by X-ray diffraction failed to unambiguously identify these two forms, probably as a result of the different nature of our low-resolution powder vis-á-vis the previous single-crystal diffraction experiments. Further complications might have stemmed from the fact that some of our powders actually entailed mixtures of polymorphs, as well as from the variability that different cocrystallized solvents (acetone, ethanol, etc.) could impart on the diffraction patterns. Still, we feel confident in assigning the powder crystallized from water at elevated temperatures to the "wet" literature B_{12} form on the basis of the following: (1) the similarity in quadrupole coupling parameters between the ^{59}Co NMR results obtained from this powder (Figure 2) and the single-crystal ^{59}Co NMR data recently reported for the "wet" form by Power and co-workers ($e^2qQ/h = 27.3 \pm 0.2$ MHz);[29] (2) the fact that after extended storage periods the powder recrystallized from hot water eventually gave
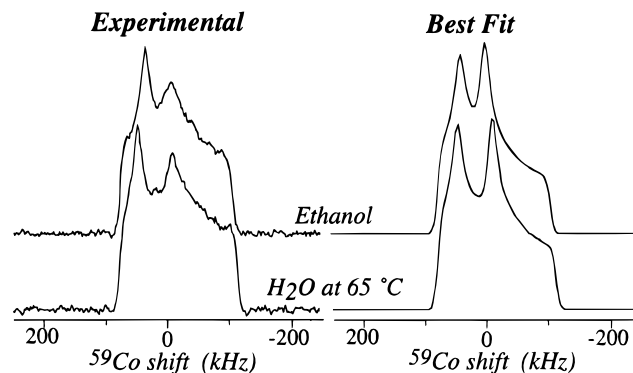
^{59}Co (and ^{13}C) solid NMR spectra similar to those samples derived from the remaining solvents (due to loss of hydration water, as reported for the "wet"-to-"dry" sample transition); and (3) the fact that all nonaqueous solvents yielded very similar spectra, suggesting the presence of a prototypical "dry" structure devoid of waters of hydration. Still, it could be argued that samples crystallized from aqueous acetone, Hodgkin's solvent of choice for obtaining the "wet" single crystals, should have yielded "wet" crystals as well. In fact, as is further detailed below such solvent yielded essentially equifractional powder mixtures of the two polymorphs, from which individual "wet"-type single crystals could easily have been extracted for the original diffraction analyses.

In view of these considerations, the differences observed by ^{59}Co NMR between the two types of crystalline environments are somewhat unexpected. Indeed the main differences reported for the two vitamin B_{12} polymorphs reside on the unit cell characteristics, dominated by the packing of cocrystallized solvent molecules residing ~10 Å away from the cobalt site.[13–15] Differences have also been detected in the conformations of one acetamide and one propionamide side chain substituting the corrinoid macrocycle, but both room and low temperature diffraction studies revealed similar structural features in the vicinity of the metal center itself for the two polymorphs.[15,17] By contrast the ^{59}Co NMR coupling parameters of the two forms differ chiefly in the quadrupole coupling constant, which measures in a local manner the electronic field gradients at the position of the metal.[32] Thus, although it cannot be ruled out that long-range effects stemming from changes in the hydration molecules or the alkylamide side chains will influence this coupling parameter, field gradients in such an asymmetric environment as that felt by the cobalt nucleus in vitamin B_{12} are almost sure to reflect the immediate coordination of the metal. To get further insight into the differences affecting this parameter in the two polymorphs a series of variable-

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temperature ^{59}Co NMR measurements were made. These powder spectra show significant changes with temperature (Supporting Information), yet line shape simulations reveal that these changes can largely be accounted for by adjusting the chemical shift (but not the quadrupole) tensor parameters. Since ^{59}Co shift parameters are known to be highly sensitive to temperature even in the absence of structural changes due to an abundance of low-lying excited states involving the metal's atomic orbitals,[33] few structural conclusions can be deduced at this point from these changes. More informative in this respect were the spin-1/2 studies of the polymorphs, whose results are summarized below.

^{13}C NMR Data. Figure 3 shows a series of room temperature high-resolution ^{13}C NMR spectra collected on powdered vitamin B₁₂ samples obtained under different crystallization conditions. As in the case of ^{59}Co NMR these spectra evidence again three main different kinds of patterns: one involving relatively broad resonances arising from "as-purchased" or rapidly evaporated samples, another observed on crystallizing the vitamin from H₂O at 70 °C, and a third one arising from room temperature crystallizations on most other solvents. A departure from this trend is only observed when the vitamin sample is slowly crystallized from aqueous acetone; the resulting powder yields a more complex ^{13}C NMR spectrum that very closely matches a 50/50 mixture of the two other ("wet" and "air-dried") crystalline samples. Such a mixture spectrum and a ^{13}C trace taken for the vitamin in an aqueous (90% H₂O/10% D₂O) solution are also shown for completion in the same figure.

A couple of features stand out upon inspecting these ^{13}C CPMAS solid spectra. One of these is the almost solution-like sharpness of the peaks arising from the recrystallized forms. Their average line widths are only ∼0.25 ppm, indicative of a very high and homogeneous crystallinity throughout the powders. Remarkable in this respect are the minor residual dipolar broadening effects that the numerous nitrogens in the molecule have despite the moderate magnetic field that was used;[34] only the benzimidazole carbon peak at 143.3 ppm, arising from a site that is flanked by two ^{14}N atoms, shows a 0.5 ppm width. This sharpness is probably a consequence of the closeness to the 125.3° (180−54.7°) angle that most C−N bonds subtend with respect to the main direction of the nitrogen's electric field gradient tensor (parallel to the latter's lone pair of electrons), a situation for which the residual ^{13}C−^{14}N MAS dipolar coupling scales considerably or entirely vanish.[35] Another potential explanation for the sharpness of the various ^{13}C lines could reside in the presence of fast segmental motions of the molecule in the solid, but this option was ruled out on the basis of 2D ^{13}C separate local field measurements[36−38] which showed essentially motionless C−H sideband manifolds for the numerous methine sites that were resolved (Supporting Information). In contrast to these highly resolved polycrystalline ^{13}C spectra stand the much broader (≈1.5 ppm) lines arising from the "as-purchased" sample. To try pinpointing the origin of these different broadenings, the T_2 values for sites in all the different samples were measured using rotor-synchronized Carr−Purcell Meiboom−Gill experiments. These indicated similar natural ^{13}C
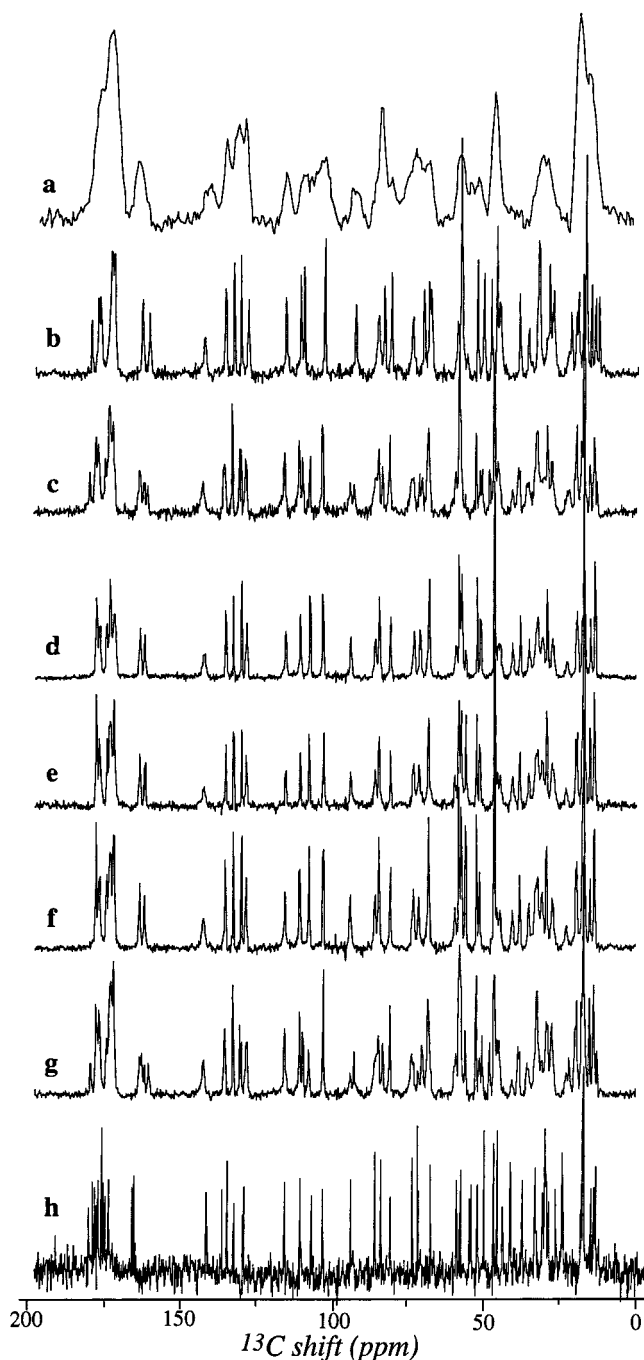
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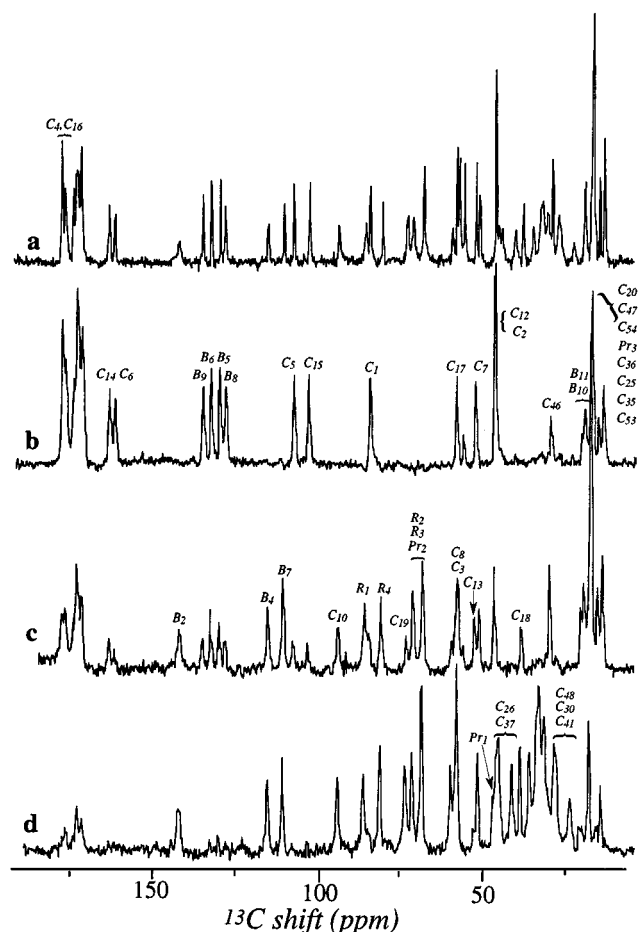(38) McElheny, D.; deVita, E.; Frydman, L. 1999, *J. Magn. Reson.*, in press.

Figure 3. 75.8 MHz (7.1 T) room temperature ^{13}C CPMAS NMR spectra acquired on a series of vitamin B₁₂ powders obtained as follows: (a) as-purchased sample; (b) after slow evaporation of an aqueous solution at 70 °C; (c) after slow room-temperature crystallization from H₂O/acetone; (d) after slow room-temperature crystallization but from isopropyl alcohol; (e) after slow room-temperature crystallization from ethanol; (f) after slow room-temperature crystallization from methanol; and (g) from a 50/50 w/w mixture of the powders used in (b) and (e). For completion, (h) shows the {^{1}H}^{13}C trace of the complex in an aqueous solution.

line width values for both the quickly evaporated and the slowly crystallized solids, ranging in all cases between 2 and 6 Hz. This in turn implies that the much wider lines observed in the "as-purchased" CPMAS spectra are a consequence of nonspecific conformational heterogeneities and not, for instance, the result of a slight paramagnetism arising from Co(II) centers.

Another noticeable feature that arises upon comparing the spectra from the two polycrystalline forms is the very localized

Figure 4. 75.8 MHz room temperature ^{13}C CPMAS NMR spectra acquired from an ethanol-crystallized vitamin B_{12} powder using (a) a regular cross-polarization sequence (all sites); (b) regular CP followed by a period of dipolar dephasing (methyl and nonprotonated carbons only); (c) an INEPT-based spectral editing (methyl, methine, and nonprotonated carbons, not necessarily with the same relative intensities as in (b)[40]); and (d) CP with a short contact time (mainly methine and methylene sites). The chemical origins of quaternary/methyl (b), methine (c), and methylene (d) sites are indicated based on solution-state assignments.[16] The approximate number of scans used in each of these acquisitions were 1000, 10000, 20000, and 4000, respectively.

Figure 5. Molecular structure of vitamin B_{12} depicting in bold characters those carbon sites for which solid-state ^{13}C NMR shows peak shifts upon going from the "wet" to the "air-dried" forms. More ambiguous however are the exact positions of the side chain carbons that also shift between polymorphs.

nature of their differences. This is particularly evident when comparing the ^{13}C spectra from the "wet" and "dry" samples with that arising from the H_2O/acetone polymorphs mixture: it is then clear that several of the polymorphs' resonances overlap exactly with one another despite their sharpness, while others are shifted by up to ≈3 ppm. Assigning the chemical origin of these shifted resonances can thus yield a perspective about the differences between the two forms of vitamin B_{12}, viewed not from a standpoint of geometrical packing or of differences in their solvents of cocrystallization but according to the changes felt by the electronic environments of each carbon site in the molecule. As aids in assigning these changes in the solid state we used the generally similar positions that several of the resonances exhibited when compared with their solution state counterpart, which have been assigned entirely during recent years using modern multidimensional NMR methods.[23,25] Also useful were the results from spectral editing techniques based on solid-state dipolar couplings, whose application toward assigning the ^{13}C spectra is exemplified in Figure 4 for one of the polymorphs. Unfortunately a combination of peak line widths, solution-/solid-phase differences, and closeness among the resonances still prevents the complete unambiguous assign-
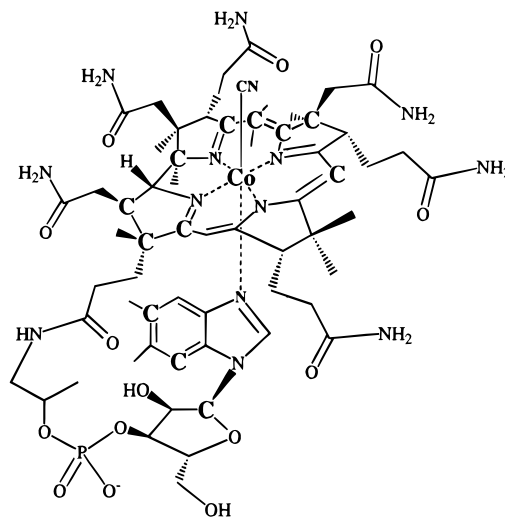
ment of all individual ^{13}C CPMAS NMR peaks; further efforts in this area are currently in progress.

Despite this shortcoming, the solid NMR spectra enable the identification of a number of sites that experience substantially different environments depending on whether vitamin B_{12} crystallized as one of the polymorphs or the other. Some of these, like the variations displayed by the low-field carbonyl (170−180 ppm) and the various methylenic (∼45, 35, and 30 ppm) regions, are to be expected on the basis of the different geometries that X-ray diffraction observes for certain acetamide and propionamide side chains. But there are also other, previously unnoticed differences between the two forms that involve sites which are much closer to the metal center itself; from low to high field and following the notation of Scheme 1 these include the corrinoid ^{13}C peaks of sites C_4 and/or C_{16}, C_6, C_5, C_{10}, C_1, C_{17}, C_7, and C_{18}, as well as resonances arising from B_5, B_7, and R_1. The positions of these affected sites within the molecular structure are indicated for further clarity in Figure 5. These involve not only branching carbons for the side chains but also such sites as C_{10}, which has been postulated as a likely candidate to be affected in butterfly-like flexing distortions of the macrocycle.[8,15,18] Also, it is clear that conformational variations at these ^{13}C-shifted positions could explain the polymorphic changes observed in the electronic field gradients of ^{59}Co NMR much more readily than the more distant side chain packing or hydration variations detected by crystallography.

Also interesting to explore is the behavior of the different polymorphs with temperature, as recent cryodiffraction analyses of the "wet" B_{12} form revealed differences when compared with the room temperature results of Hodgkin and co-workers.[17] These differences were again confined mainly to the motional status and/or the position of cocrystallized solvent molecules; the structures of the B_{12} molecules themselves, however, showed up essentially equal or with variations within experimental error at the two temperatures. By contrast, solid-state ^{13}C NMR reveals spectra that for both polymorphs are temperature dependent (Figure 6). Because no true motional dynamics could be detected in the separate-local-field spectra of these compounds, these changes are probably reflecting the temperature dependence of the forms' crystal habits. This in turn can affect
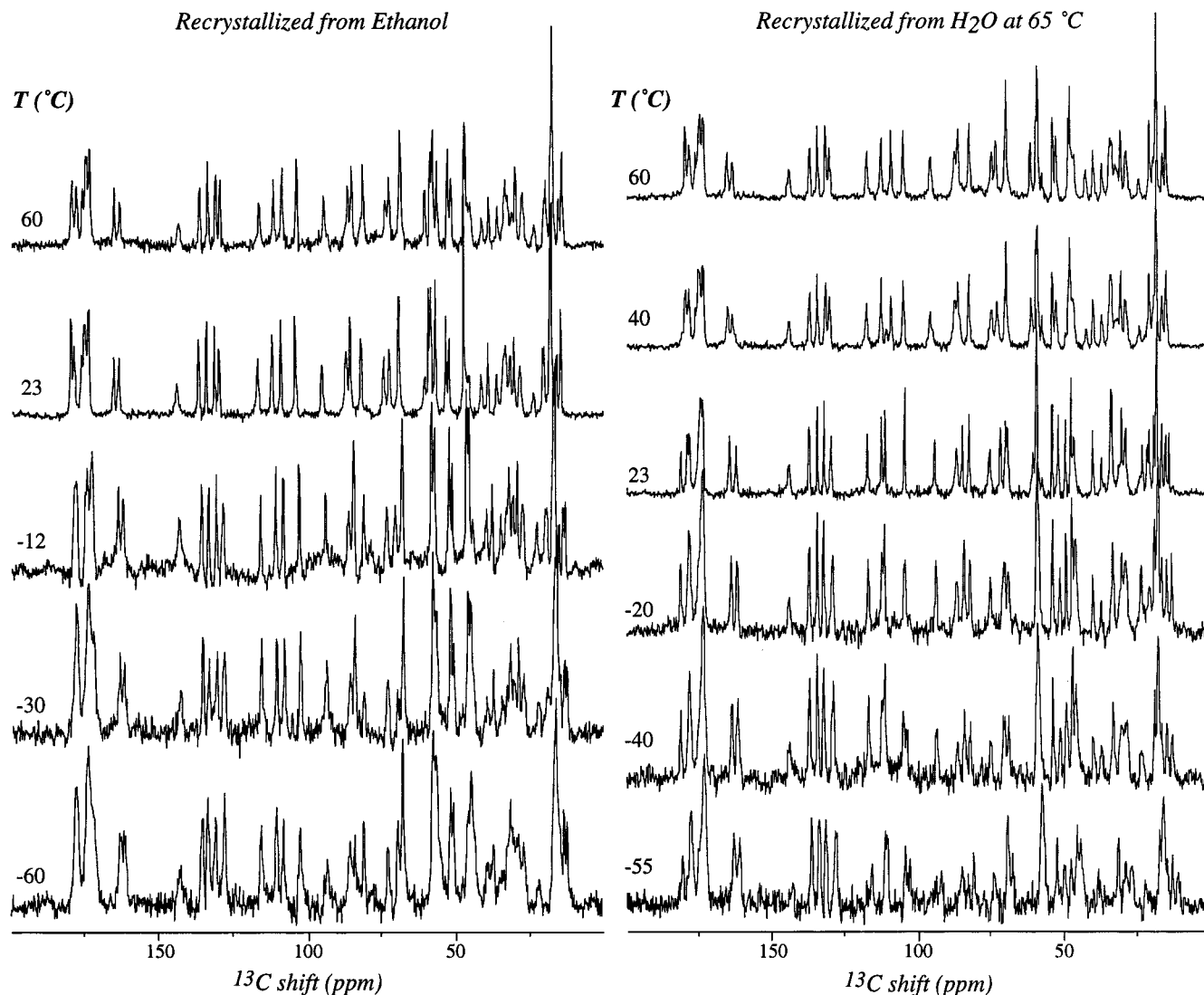
Figure 6. Variable-temperature ^{13}C CPMAS NMR spectra acquired from vitamin B$_{12}$ powders crystallized under the indicated conditions, showing specific (and reversible) shifts in the resonances. Changes in signal-to-noise may stem from lengthened proton relaxation times and therefore are unspecific; at all temperatures the MAS rates were kept above 7.5 kHz for the sake of minimizing spinning sidebands.

the chemical shifts of vitamin B$_{12}$ via molecular distortions introduced by the changing crystal packing forces. Supporting this hypothesis is the reversible nature of the observed spectral changes over the entire temperature range that was explored. Also interesting to note is the fact that not all resonances are equally affected by temperature, and those which are most strongly affected correspond closely to the peaks that showed the biggest displacements upon going from one of the crystalline forms to the other. This suggests that these resonances correspond to sites possessing the largest structural flexibility in the macrocycle, prone to give in upon changing the external packing conditions of the crystalline environment.

^{15}N and ^{31}P MAS NMR Data. In an effort to enrich the insight revealed by these ^{59}Co and ^{13}C NMR data, CPMAS ^{15}N and ^{31}P NMR spectra were also recorded at natural abundance for the two vitamin B$_{12}$ polymorphs. Some of these data are illustrated in Figures 7 and 8, respectively. From the former it appears that not all nitrogen sites are observable in the ^{15}N CPMAS experiments: on comparing the polymorphs results with the assigned ^{15}N NMR spectra available for the vitamin in solution,[24,27] it would seem that only the six amide sites and the benzimidazol site NB$_1$ contribute to the solid line shape. The absence of the remaining five nitrogens even after assaying

a variety of contact times (1−15 ms long) suggests a strong residual coupling between these "missing" sites and the cobalt atom to which they are all directly bonded; indeed, a recent cobaloxime−(^{15}N)pyridine solid-state NMR study has shown that such couplings can exceed 500 Hz,[39] and for our natural abundance cobalamins this could easily broaden signals beyond the limit of detection. Still, obvious differences can be appreciated both between the polymorphs ^{15}N NMR spectra as well as with respect to the solution expectation. By contrast, the ^{31}P NMR spectra arising from the phosphate groups of the crystalline polymorphs are virtually indistinguishable from one another; not even a line width increase in the single phosphate resonance is observed upon comparing the "wet" and "dry" form spectra with that of the mixture known to arise from aqueous acetone crystallization (Figure 8). The half width of all these phosphorus resonances is ∼0.25 ppm, significantly narrower than the ∼5 ppm half width associated with the resonance of the "as-purchased" sample. As in the case of the ^{13}C spectra, a ^{31}P spin−echo experiment revealed that the homogeneous line width for this peak is actually ca. 10 times smaller than that observed by simple 1D excitation. Other interesting features stemming from

(39) Schurko, R.; Wasylishen, R. Personal communication.
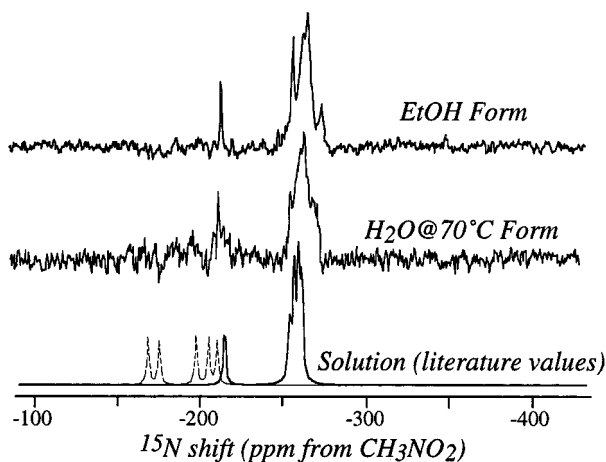(40) deVita, E.; Frydman, L. Manuscript in preparation.

Figure 7. Comparison between the room temperature natural-abundance ^{15}N CPMAS NMR spectra acquired from the two polymorphs of vitamin B_{12} at 30.6 MHz, and the ^{15}N NMR spectrum expected on the basis of the nitrogen's chemical shifts as reported in the literature. For the latter it was assumed that all sites had identical spectral intensities and all lines were 1.5 ppm wide; the dotted resonances arise from the five sites that are directly bonded to the cobalt atom, apparently absent (or considerably broadened) in the solid traces. The experimental spectra result from 50000 signal averaging scans and are externally referenced to nitromethane using solid ammonium sulfate as external standard.
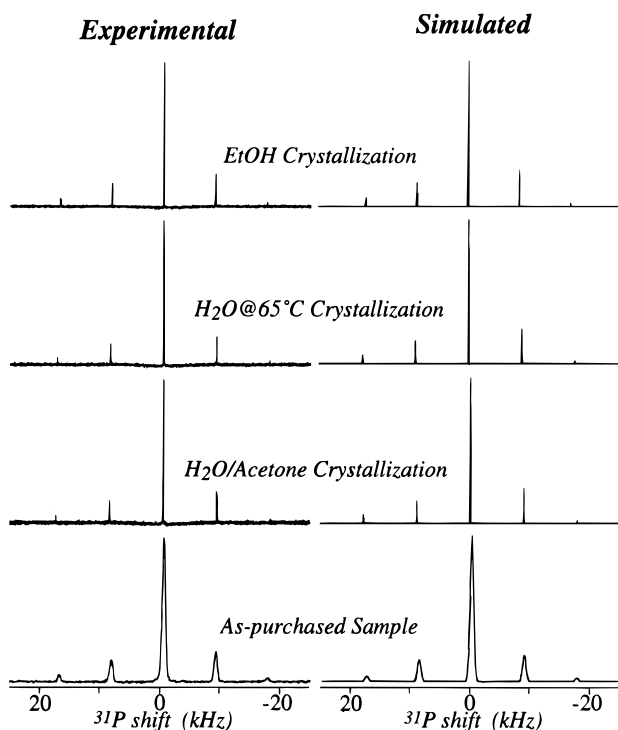

Figure 8. Comparison between the 122.1 MHz room temperature ^{31}P CPMAS NMR spectra acquired from the indicated vitamin B_{12} samples (32 scans) and simulations calculated for the sake of characterizing the chemical shift tensor parameters (only data in the 8.7−8.9 kHz spinning range shown). According to the fits the phosphates in all forms have isotropic shifts of −8.0 ± 1 ppm downfield from 85% H_3PO_4 (as indicated by a triphenylphosphine secondary standard), shift anisotropies of $\delta_{CS} = 119 \pm 4$ ppm for the crystalline forms and 105 ± 5 ppm for the as-purchased one, and asymmetry parameters of 0.3 in all cases.

these ^{31}P NMR experiments are shielding anisotropies that are comparable to those observed in monophosphate nucleotides[41] and a weak temperature dependence of the isotropic shifts (~0.08 ppm per deg, Supporting Information).

Conclusions

Understanding the connection between cobalamins' structures and their distinct enzymatic role as carriers of organic radicals has led to a long and fruitful search.[2,3,5,6] Central to this quest has been elucidating Nature's rationale for preferring the biosynthetically more "elaborate" corrinoid structures over "simpler" porphyrinoid derivatives used in many other natural functions.[7] As noted by several researchers a central difference between cobalamin and porphyrin structures rests in the former's ability to flex their corrinoids in a "butterfly"-like mode, thanks to the reduction and elimination of certain macrocyclic bonds. This in turn would provide enzymatic reactions with the possibility to trigger structurally the dissociation of the carbon−cobalt bond by driving an upward folding of the in-plane ligand. Following this premise numerous studies have looked for and found systematic correlations between the metal−axial ligand bond distance and in-plane ligand distortions, both in cobalamins and in simpler organocobalt model compounds.[14,15,17] Still, because in a majority of these studies such correlations were brought about by changing the chemical coordination surrounding the metal, it may be hard to unambiguously discriminate in them what proportion of the structural variations were due to steric effects and how much resulted from the electronic changes. It is known on the other hand that dealing with polymorphs offers a way out from this dicotomy, since in these cases no chemical variations are involved and all that is at play are steric interactions between the crystal packing forces and the molecular conformation. In this sense the present solid-state NMR investigation offers another good possibility of experimentally exploring the "flexible" points in the cobalamin structure, by pinpointing the sites whose peaks shift from one polymorph to the other as a result of changes in packing. The fact that many of these peaks were found to involve not only positions in the conformationally flexible side chains but also sites in the macrocycles themselves provides a chemically unbiased perspective regarding the type of deformations that these structures can undergo. In this respect it is also worth mentioning that although polymorphic ^{13}C or ^{15}N shifts in molecules as large as vitamin B_{12} are not unexpected, these have not to our knowledge been reported for macrocyclic sites in the many porphyrin and phthallocyanine derivatives that have hitherto been studied.

We believe that this work presents but a few of the potential applications that solid-state NMR could have to the structural analysis of vitamin B_{12} and its derivatives. Further progress could be achieved by employing higher magnetic fields capable of better resolving the signals and thus making them more amenable to a complete assignment analysis; at least partial isotopic enrichment should also facilitate studies on holoenzymatic systems and the incorporation of NMR-based distance measurements between specific sites in the complex. Also worth incorporating are theoretical calculations that could help clarify the origin of the various spectral changes on the basis of the available polymorphic structures; these research avenues are currently being pursued.

(41) Terao, T.; Matsui, S.; Akasaka, K. *J. Am. Chem. Soc.* **1977**, *99*, 6136.

Supporting Information Available: Figure A: variable-temperature ^{59}Co NMR line shapes acquired from an ethanol-recrystallized vitamin B$_{12}$ sample. Figure B: comparison between selected ^{1}H$-^{13}$C MAS sideband patterns. Figure C: temperature dependencies observed for the isotropic ^{31}P CPMAS NMR centerbands of the various vitamin B$_{12}$ powders indicated in Figure 8 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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