Identification of Structural Markers for Vitamin B_{12} and Other Corrinoid Derivatives in Solution Using FTIR Spectroscopy†

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ABSTRACT: The identification of structural markers for B_{12}/protein interactions is crucial to a complete understanding of vitamin B_{12} transport and metabolic reaction mechanisms of B_{12} coenzymes. Fourier transform infrared spectroscopy can provide direct measurements of changes in the side chains and corrin ring resulting from B_{12}/protein interactions. Using FTIR spectroscopy in various solvent systems, we have identified structural markers for corrinoids in the physiological state. We assign the major band (denoted B), which occurs at ca. 1630 cm^{-1} in D_2O and ca. 1675 cm^{-1} in ethanol, to the amide I C=O stretching mode of the propionamide side chains of the corrin ring. The lower frequency of band B in D_2O versus ethanol is due to the greater hydrogen-bonding properties of D_2O that stabilize the charged amide resonance form. Since the propionamides are known to be important in protein binding, band B is a suitable marker for monitoring the interaction of these side chains with proteins. We assign bands at ca. 1575 and 1545 cm^{-1} (denoted C and D) as breathing modes of the corrin ring on the basis of the bands' solvent independence and their sensitivity to changes in axial ligation. As the σ-donating strength of the axial ligands increases, the frequencies of bands C and D decrease, possibly indicating a lengthening of the corrin conjugated system. Band A, the known cyanide stretching frequency at ca. 2130 cm^{-1}, probes the cobalt–carbon distance in cyanocorrinoids. As the frequency of band A increases, the cobalt–carbon bond strength should decrease.

Vitamin B_{12} coenzymes are essential cofactors in enzymatic reactions of mammalian metabolism, including the conversion of methylmalonyl-CoA to succinyl-CoA and the synthesis of methionine from homocysteine. Cyanocobalamin is inactive in catalysis and must be converted to an active coenzyme form, which has 5′-deoxyadenosyl or methyl as the sixth ligand. However, the exact mechanism by which enzymes bind the coenzyme and promote cobalt–carbon bond cleavage is unknown. Infrared spectroscopy can be a useful tool for probing B_{12}/protein interactions. Previous IR studies of cobalamins primarily focused on changes in the C–N stretch band (approximately 2130 cm^{-1}) with respect to the trans axial ligand (Firth et al., 1968). The IR work of Hogenkamp et al. (1965) and the later work of Rajoria and Nath (1977) called attention to the rich IR spectra of cobalamins but failed to adequately assign many bands of interest. Furthermore, these IR studies were carried out in the solid state, i.e., KBr pellets, and are not relevant to studying B_{12}/protein interactions.

A number of cobalamin structures have been determined by X-ray crystallography, and accurate structures exist for methylcobalamin and adenosylcobalamin (Rossi et al., 1985; Savage et al., 1987). The overall bond connectivity is shown in Figure 1. All cobalamins contain a cobalt atom bound equatorially to four nitrogens in a tetrapyrrole system called the corrin ring, which contains six conjugated double bonds. On the periphery of the corrin ring there are six primary amide groups—three acetamide chains (a, c, and g) extending toward the upper (sixth) ligand and three propionamide chains (b, d, and e) extending toward the lower (fifth) ligand. The lower axial ligand of cobalamins is a 5,6-dimethylbenzimidazole (DMB)[1] group that is connected by a nucleotide moiety to a fourth propionamide side chain (f) of the corrin ring.

UV and circular dichroic spectra have been used in an attempt to monitor the changes in B_{12} structure during protein binding. Cobalamins have characteristic UV and circular dichroic spectra that are consistently altered when they are bound to cobalamin-binding proteins or cobalamin-dependent enzymes. These changes reflect the effect of B_{12}/protein interactions since the proteins have no absorption between 300 and 600 nm (Babior & Li, 1969; Nexo & Olesen, 1976). In general, the major UV band at 350–370 nm increases in wavelength and intensity when the cobalamins are bound to protein. Nexo and Olesen (1982) interpret the increase in intensity as an increase in symmetry around the cobalt atom. The increase in the negative ellipticity in the CD spectra around 540 nm for protein-bound cobalamins (including enzymes) could also indicate that the corrin ring is in a more rigid state (Babior & Li, 1969; Nexo & Olesen, 1982). Unfortunately, these UV and CD studies of free cobalamins versus protein-bound cobalamins do not provide unambiguous information about changes in the cobalamin structure upon binding, although they do suggest that changes occur.

In order to study the role of the corrin ring side chains in B_{12} interactions with diol dehydrase, Toraya et al. (1979) derivatized the primary propionamide side chains of the corrin ring of adenosylcobalamin with –COOH, $-COOCH_3$, and $-CONHCH_3$ groups. Since modification of each primary propionamide group reduces catalytic activity, all these amides contribute to formation of the active holoenzyme complex. On the basis of the relative order of the analogues' activity, Toraya

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[1] Abbreviations: DMB, 5,6-dimethylbenzimidazole; fwhm, full-width half-maximum.

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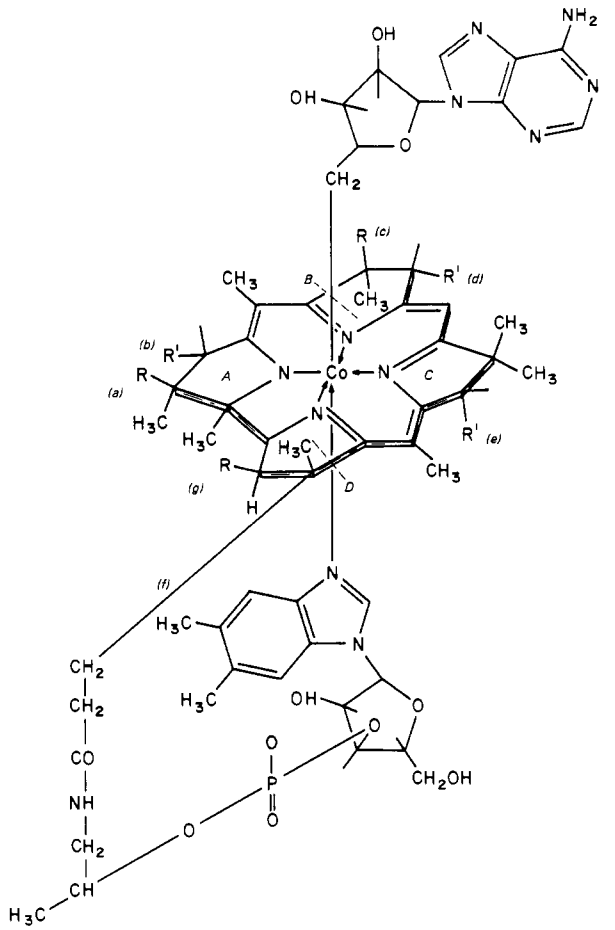


FIGURE 1: Structure of adenosylcobalamin. The acetamide side chains (a, c, and g) of the corrin ring are denoted by R. The propionamide side chains (b, d, e, and f), which are most easily hydrolyzed, are denoted with R′. A–D denotes the pyrrole rings that comprise the conjugated corrin ring system. Cobalamin compounds are distinguished by the identity of the sixth axial ligand. For adenosylcobalamin the sixth ligand is 5′-deoxyadenosyl. If this site is occupied by a cyanide group, the compound is called cyanocobalamin (vitamin B_{12}), and if occupied by water it is known as aquocobalamin.

et al. (1979) concluded that steric effects play a role in the interactions with the d side chain, hydrogen bonding seems to be important at the b side chain, and the e side chain seems to be affected by both steric effects and hydrogen-bonding ability. However, the information about the B_{12} binding site is obtained indirectly, i.e., by observing the consequences of substitution of the propionamide side chains. Furthermore, the interpretation of these results depends on the correct assignment of the isomers.

Modern 2D NMR has been the first method to provide unambiguous structural assignments for the side chains of isolated cobalamins (Pagano & Marzilli, 1989). ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence experiments detect long-range ^{1}H–^{13}C coupling, allowing for assignment of carbon resonances in the side chains. Carboxyl groups generated by amide hydrolysis can be assigned by the shift between the protonated and deprotonated forms. The unambiguous assignment of the cyanocobalamin b and e monocarboxylic acid isomers has led to the reinterpretation of studies prior to 1980, including the diol dehydrase study by Toraya (the assignments given above are correct on the basis of recent NMR data). Although NMR spectroscopy provides the correct assignment of the corrin ring side chains, the large molecular masses of B_{12} protein complexes make comparable protein studies difficult. IR spectroscopy offers the possibility of directly observing side-chain and corrin ring modes even in a holoenzyme.

Recent FT-Raman spectroscopy of cobalamins in solid form and cobaloximes [cobalt(III) complexes with a dimethylglyoxime equatorial moiety] in solid and solution have definitively identified the cobalt–carbon stretching frequency (Nie et al., 1989, 1990a,b) and are complementary to the studies conducted here. These studies also identify modes of the equatorial ligand but do not report as wide a range of compounds as seen here.

The preceding makes it clear that the amide side chains and the corrin ring are key structures involved in the mediation of both B_{12}/transport protein and B_{12}/enzyme interactions. We report solution FTIR results that assign specific bands in the 1500–1700-cm^{-1} region to corrin ring and amide structures. The former can be seen in both D_2O and 75% glycerol, while the amide band can be seen in D_2O. We show that these markers are sufficiently intense to be observed at $\leq$1 mM concentration, which is feasible for future protein studies. Therefore, these markers can be used to monitor B_{12}/protein interactions in the physiological state.

Experimental Procedures

IR spectra were measured on a MIDAC Model 101025 tabletop spectrometer with a DTGS detector. All spectra were recorded with 2-cm^{-1} resolution and were the average of 256 scans. The quoted peak positions are accurate to $\leq$1 cm^{-1} on the basis of reproducibility and spectral signal/noise. Both the interferometer and the sample chamber were extensively purged with dry nitrogen to decrease the contributions of water vapor absorbance. Adenosylcobalamin, dicyanocobinamide, hydroxocobalamin, methylcobalamin, and cyanocobalamin were purchased from Sigma; L-glutamine and 5,6-dimethylbenzimidazole were purchased from Aldrich. All samples were used without further purification. D_2O (99.9%), 100% ethanol, and 75% glycerol were used as solvents. The liquid samples were 6–10 mM in concentration depending on solubility. The samples were placed in a Spectra-Tech liquid cell equipped with CaF_2 windows and a 100-μm path length Teflon spacer. A spectrum of the appropriate solvent was used as the reference.

The pK of the protonation of the coordinated nitrogen of DMB is approximately 3.5 for adenosylcobalamin and 2.7 for methylcobalamin (Pratt, 1972). Upon protonation, DMB is detached from cobalt; thus the form with the displaced DMB is referred to as "base-off". The yellow base-off forms of adenosylcobalamin and methylcobalamin were formed by acidifying an 8 mM solution of the respective red cobalamin. Spectra were taken in D_2O at pH = 7.0, where the sample is entirely "base-on", and at 1-pH-unit intervals down to pH = 1.0. Phosphate buffer (200 mM) was used at pHs 1, 2, 3, 6, and 7. Phthalate buffer (100 mM) was used at pHs 4, 5, and 6. Co(II) B_{12} was generated by anaerobic photolysis of 10 mM adenosylcobalamin in D_2O. Optical spectra were taken in an HP 8452 diode array spectrophotometer in the IR sample holder to verify the sample composition immediately before and after IR experiments. The appearance of bands at 458 and 376 nm and at 460 and 374 nm indicates the formation of base-off adenosylcobalamin and base-off methylcobalamin, respectively (Chemaly & Pratt, 1980); the appearance of bands at 473 and 312 nm indicates the formation of Co(II) B_{12} (Dolphin, 1970).

An additional spectrum of cyanocobalamin was obtained in KBr. Cyanocobalamin (5 mg) was homogenized with 300 mg of KBr by using a mortar and pestle and then pressed in a die to 20000 psi. An air spectrum was used as the reference.

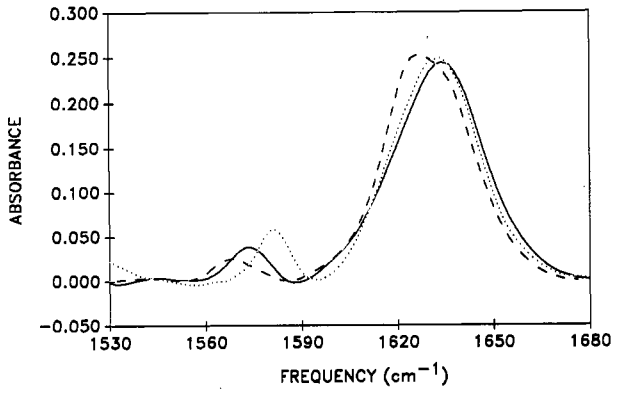


FIGURE 2: FTIR spectra of cyanocobalamin (—), adenosylcobalamin (---), and dicyanocobinamide (···) in D_2O. Spectra were collected as described under Experimental Procedures. All spectra were referenced against D_2O and baseline-corrected by using a cubic polynomial.

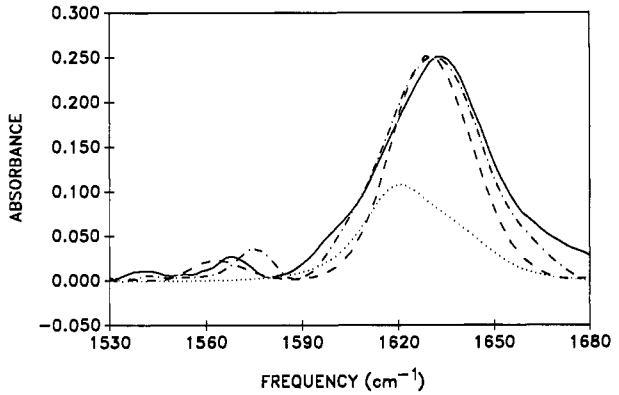


FIGURE 3: FTIR spectra of aquocobalamin (—), methylcobalamin (---), cob(II)alamin (-·-), and glutamine (···) in D_2O. Experimental conditions are described under Experimental Procedures.

Curve fits were carried out by using a Galactic Industries curve-fitting program employing log-normal (Metzler et al., 1973) and Lorentzian functions.

Results

Figure 2 shows FTIR spectra of adenosylcobalamin, cyanocobalamin, and dicyanocobinamide from 1500 to 1700 cm^{-1} in D_2O. A major band is seen at ca. 1630 cm^{-1}, with an extinction coefficient of ca. 2.6 OD units mM^{-1} cm^{-1} (band B). The band for adenosylcobalamin is asymmetric, indicating multiple components. The second most prominent band (C) is at ca. 1575 cm^{-1} and shows considerable frequency and intensity shifts for the different corrinoids. This band was originally observed in solid spectra by Rajoria and Nath (1977). A third band of very low intensity ($\epsilon \leq 0.1$ OD unit mM^{-1} cm^{-1}) is seen at ca. 1540 cm^{-1} (band D) in all the corrinoids studied. Its presence in dicyanocobinamide in D_2O is uncertain because of the small extinction of this band, yet it is observed in ethanol. Band D, as well as bands A, B, and C, is not present in high-concentration DMB spectra in D_2O (data not shown).

In Table I, the peak frequencies of the major absorption bands of corrinoids in D_2O, ethanol, 75% glycerol, and KBr are shown. Figure 3 shows the D_2O spectra of aquocobalamin, methylcobalamin, Co(II) B_{12}, and glutamine. Band B for aquocobalamin, methylcobalamin, and Co(II) B_{12} is similar to that for cyanocobalamin and dicyanocobinamide, showing no asymmetry as observed for adenosylcobalamin. Bands C and D are also observed and show some shifts depending on the compound. Glutamine, used as a primary amide group

Table I: IR Peak Frequency Data for Cobalamin and Model Compounds[a]

	A	B	C	D
D_2O				
cob(II)alamin	–[b]	1630	1563	1533
methylcobalamin	–	1632	1568	1540
adenosylcobalamin	–	1630	1570	1539
cyanocobalamin	2138	1633	1574	1544
aquocobalamin	–	1632	1575	1543
dicyanocobinamide	2122	1632	1582	1551
glutamine	–	1622	–	–
EtOH				
methylcobalamin	–	1675	1567	1540
cyanocobalamin	2134	1679	1573	1545
hydroxocobalamin	–	1676	1575	1548
dicyanocobinamide	2124	1676	1580	1552
75% glycerol				
cyanocobalamin–H_2O	2137	[c]	1573	1549
cyanocobalamin–D_2O	2138	1633	1574	1543
KBr				
cyanocobalamin	2135	1665	1572	1547

[a] All data are in wavenumbers. Base-off adenosylcobalamin and base-off methylcobalamin results are similar to base-on compounds in D_2O. [b] (–) denotes band not observed. [c] Band obscured by solvent absorbance.

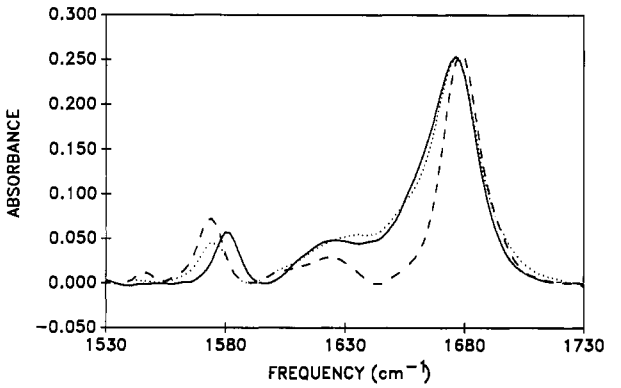


FIGURE 4: FTIR spectra of dicyanocobinamide (—), cyanocobalamin (---), and hydroxocobalamin (···) in ethanol. Experimental conditions are described under Experimental Procedures.

model, shows a large band at 1622 cm^{-1} in D_2O (ϵ = 1.1 OD units mM^{-1} cm^{-1}) and no evidence of band C or D. Figure 4 shows FTIR spectra of cyanocobalamin, dicyanocobinamide, and hydroxocobalamin in ethanol. Hydroxocobalamin is presumably converted to ethoxocobalamin in ethanol. However, on the basis of the similarity between the optical spectra of hydroxocobalamin in H_2O and D_2O versus ethanol, we term the compound hydroxocobalamin. In ethanol we observe band B for cyanocobalamin at 1679 cm^{-1} (ϵ = 2.6 OD units mM^{-1} cm^{-1}) and similar symmetric bands for the other compounds. Bands C and D are also observed for all three compounds but are not significantly shifted from their position in D_2O. Additional bands also appear at 1620 and 1590 cm^{-1} that may be masked by band B in D_2O. Clearly, band B is shifted and narrower for all spectra in ethanol compared to those in D_2O. Band B for cyanocobalamin cannot be seen in 75% glycerol–H_2O due to the solvent's absorbance, but it is visible in KBr at 1665 cm^{-1} and in 75% glycerol–D_2O at 1633 cm^{-1}.

In the methylcobalamin and adenosylcobalamin to base-off titrations, we observe only small shifts in bands B and C. Figure 5 compares the spectra of methylcobalamin (pH = 7.0) with the methylcobalamin base-off form in which DMB has been protonated and detached from the Co (pH = 1.0). At the lower pHs band C may shift to slightly higher frequencies, but any change is less than 2 cm^{-1} and is difficult to verify due to the noise level. Adenosylcobalamin gives similar results,

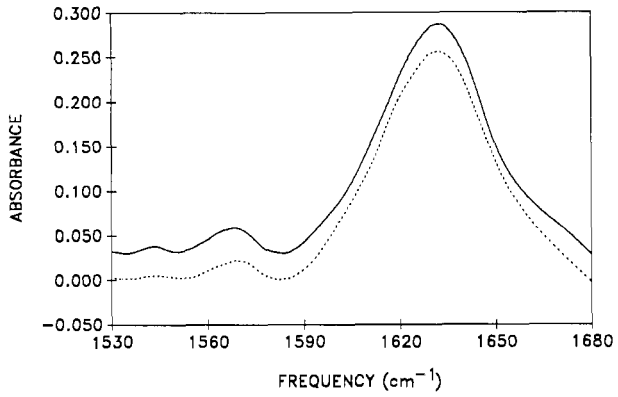


FIGURE 5: Comparison of methylcobalamin spectrum at pH = 7.0 (—) with methylcobalamin base-off spectrum at pH = 1.0 (---) in D_2O. Both spectra were obtained under the same conditions: 8 mM methylcobalamin, 200 mM phosphate buffer, 100 μm path length, 256 scans, and 2-cm^{-1} resolution. The only difference was in the pH.

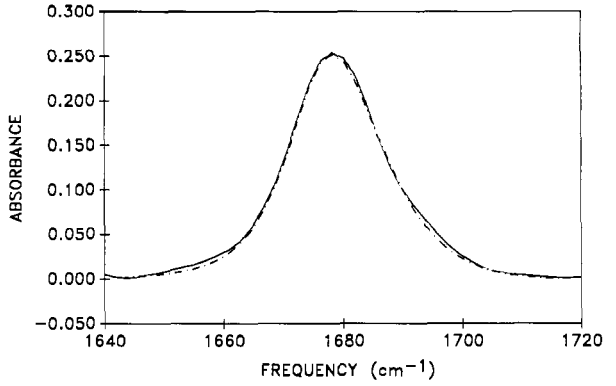


FIGURE 6: Curve fit (---) for band B of cyanocobalamin spectrum (—) in ethanol. A fit with one log-normal band and one Lorentzian band, as described in the text, is shown.

and the results for the intermediate pH values are the same.

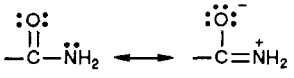
We also observe a large broad band at ca. 1450 cm^{-1} in all of the cobalamin, dicyanocobinamide, glutamine, and DMB spectra (data not shown). In D_2O the band has shoulders at 1428 and 1487 cm^{-1} for adenosylcobalamin, 1479 cm^{-1} for aquocobalamin, 1475 cm^{-1} for cyanocobalamin, 1503 cm^{-1} for dicyanocobinamide, and 1428, 1479, and 1492 cm^{-1} for methylcobalamin. In the glutamine D_2O spectrum we observe two smaller bands at 1409 and 1453 cm^{-1}. The approximately 1450-cm^{-1} band is at a known strong C–H vibrational frequency (Parker, 1983), which is a reasonable assignment for these compounds.

In the ethanol, 75% glycerol, and KBr samples we observe the C–N stretch band (A) at ca. 2130 cm^{-1}, which was assigned by earlier IR work (Firth et al., 1968). We observe this band in ethanol at 2134 cm^{-1} for cyanocobalamin and 2124 cm^{-1} for dicyanocobinamide. For cyanocobalamin, band A occurs at 2135 cm^{-1} in KBr and at 2137 cm^{-1} in 75% glycerol. In D_2O, band A appears at 2122 cm^{-1} for dicyanocobinamide and at 2138 cm^{-1} for cyanocobalamin.

Discussion

Our data identify several IR bands of varying intensity in the first extensive study of cobalamin compounds in solution. In Table I, the main transitions and their frequencies are summarized. Band A, as mentioned above, is the C–N stretch band of cyanocorrinoids and is present only for the cyano compounds. Band C was previously suggested to be a corrin ring mode, and our results support this assignment. Bands B and D have not been previously identified, and their assignment is discussed below.

The amide I C=O stretching mode is generally observed in the 1620–1690-cm^{-1} region for a primary amide (Parker, 1983). We assign band B to the acetamide and propionamide side chains since its frequency is characteristic of a primary amide I C=O stretching mode. The frequency of the amide I band is approximately 50 cm^{-1} lower in D_2O than in ethanol for dicyanocobinamide, methylcobalamin, and cyanocobalamin. The effect of solvent hydrogen bonding explains the differences in amide I frequency for the different solvents. For a primary amide, the following resonance forms are possible:



The actual structure is intermediate between the two above forms depending on solution conditions. A highly polar solvent such as D_2O increases the stability of a form that is closer to the $C–O^-$ form, and the corresponding increase in single-bond character results in a lower IR frequency for the carbonyl group. In the D_2O spectrum of glutamine, band B occurs at 1622 cm^{-1}, a frequency lower than those observed for the corrinoids. The decrease in frequency is a result of favorable intramolecular interactions between the charged resonance form and the charged amino and carboxyl groups of the amino acid that further stabilize an intermediate close to the $C–O^-$ form.

Band B for cyanocobalamin (and the other compounds) is clearly narrower in ethanol than in D_2O. However, even in ethanol the band width of 17 cm^{-1} fwhm is not characteristic of a homogeneous environment. This result is not surprising, since the spectrum is a combination of one secondary and six primary amides. The amide I mode for secondary amides is generally 10 cm^{-1} higher in frequency than for primary amides of the same environment (Parker, 1983). As a result, fits to band B in ethanol using single Lorentzian, or log-normal curves (Metzler et al., 1973) were unsuccessful. Fits with two symmetric bands were also inadequate. The fit shown in Figure 6 represents the minimum number of free parameters necessary to fit band B. A combination of an asymmetric 16 cm^{-1} fwhm log-normal band at 1679 cm^{-1} (ρ = 1.42) and a Lorentzian band of 13 cm^{-1} fwhm at 1669 cm^{-1} are required. This analysis suggests a heterogeneous hydrogen-bonding environment for the different amides. The spectrum of cyanocobalamin in D_2O is considerably wider than in ethanol, with a fwhm of 33 cm^{-1}. In Figure 4, a number of bands are seen from 1590 to 1640 cm^{-1}; however, these bands are insufficient to fully account for the greater width of band B observed in D_2O. Thus, the widths for all the D_2O spectra are due to a much greater heterogeneity of the hydrogen-bonding environment than in ethanol. All the spectra of bands C and D are adequately fit by single Lorentzian components of 9–10 cm^{-1} fwhm, indicating that homogeneous broadening is dominant.

Since the propionamide side chains are known to be important in protein binding, band B is a suitable marker for monitoring the interactions of the side chains of cobalamins with proteins. Specifically, properly oriented hydrogen-bond donors of the protein will stabilize the $C–O^-$ structure while hydrogen-bond acceptors will stabilize the $=NH_2^+$ structure. In this situation, band B will shift to a lower frequency (as with glutamine) so that it may be resolved from the secondary amide modes of proteins observed from 1630 to 1700 cm^{-1} (Parker, 1983; Mantsch et al., 1988).

We assign the approximately 1575 cm^{-1} band (C) as a corrin ring mode. It is virtually independent of solvent, exhibiting only small shifts for dicyanocobinamide, methylcobalamin, and

cyanocobalamin in D_2O versus ethanol. Absorbance at this frequency might be due to the amide II N–H deformation mode, which is observed from 1590 to 1620 cm^{-1} for primary amides in H_2O. However, in D_2O this mode is shifted below 1500 cm^{-1} (Parker, 1983). Furthermore, if band C is an amide II mode, it would be present in the glutamine spectrum and also exhibit a large shift for different solvents. The observation of this band at approximately the same wavelength in solid spectra is also inconsistent with an amide II assignment. Band D is also likely to be a corrin ring mode on the basis of its similarities to band C. Band D exhibits only slight changes with respect to solvent; however, its intensity varies significantly. The changes in frequencies of band D for different cobalamins are similar to those in band C. Unfortunately, its extinction coefficient is low. Since it is not observed in spectra of pure DMB and is found in spectra of cobalamins and dicyanocobinamide, it is not a mode of DMB.

The variations in band C with cobalamin structure are consistent with a corrin ring assignment. Band C is solvent independent but is sensitive to cobalamin structure. Furthermore, the changes in frequency of this band are not solely due to steric effects of the axial ligands. There is a 7-cm^{-1} difference in the frequency of this mode between methylcobalamin and aquocobalamin, which have very similar ligand sizes. Yet there is only a 2-cm^{-1} difference between methylcobalamin and adenosylcobalamin, which have ligands of distinctly different sizes but similar electronic properties and structure at the cobalt ion (Savage et al., 1987; Rossi et al., 1985; Sagi et al., 1990).

Bands C and D may reflect changes in the structure of the corrin conjugated system as influenced by the differences in electronic structure of the axial ligands. The axial ligands in Table I have σ-donor strengths in the following order: $-CH_3$ > 5′-deoxyadenosyl > $-CN$ > H_2O (Pratt, 1972). Alkyl ligands such as $-CH_3$ and 5′-deoxyadenosyl donate electrons to the cobalt coordination system, lengthening the cis and trans bonds. On the other hand, an electronegative ligand such as $-CN$ withdraws electrons from the cobalt and, thus, shortens the bonds to the cis and trans ligands. Comparing the cobalamins (corrinoids with a lower axial DMB ligand), the frequency of the corrin ring mode in D_2O increases from 1568 cm^{-1} for methylcobalamin to 1570 cm^{-1} for adenosylcobalamin, to 1574 cm^{-1} for cyanocobalamin, and to 1575 cm^{-1} for aquocobalamin. The trend for band D is essentially the same. As the σ-donor capacity of the upper axial ligand increases, there is a decrease in the frequency, possibly indicating a lengthening of the corrin conjugated structure. For Co(II) B_{12}, which is fully reduced by 1 electron equiv compared to the above compounds, these frequencies are even lower: 1563 cm^{-1} for band C and 1533 cm^{-1} for band D. The lower frequencies indicate even greater donation of electron density to the corrin conjugated system and a lengthening of the vibrational unit to accommodate this density. In the methylcobalamin and adenosylcobalamin to base-off titrations, we observe minimal changes in the corrin ring mode. Despite the presumed shift to a five-coordinate species (Chemaly & Pratt, 1980), the very small change in band C indicates little rearrangement of electron density upon cleavage of the cobalt–nitrogen bond. However, this is reasonable considering that this bond is very weak.

Previous resonance Raman spectra provide further support for the corrin ring mode assignment of bands C and D (Mayer et al., 1973; Salama & Spiro, 1977). These spectra identify a strongly resonance enhanced vibrational mode at 1500–1550 cm^{-1} (depending on excitation frequency) for corrinoid compounds. This mode is presumed to reflect breathing motions of the corrin ring. A disappointing feature of these results is the small (<3 cm^{-1}) shifts in the vibrational frequencies as a function of ligand. Therefore, resonance Raman spectroscopy is not very useful in characterizing changes in corrin ring structure.

Nie et al. (1990a), using FT-Raman spectroscopy, have observed a number of presumptive corrin ring modes in solid spectra of methylcobalamin, cyanocobalamin, and adenosylcobalamin. Although the resolution of their published spectra is poor, two bands, apparently identical with bands C and D observed here, are seen at 1575 and 1545 cm^{-1} in cyanocobalamin, 1567 and 1540 cm^{-1} in adenosylcobalamin, and 1565 and 1538 cm^{-1} in methylcobalamin. These results are entirely consistent with the results observed here. Their results also suggest that the structure corresponding to these bands is very similar in the solid versus liquid states, unlike the cobalt–carbon bond frequency of cobaloximes, which changes dramatically from solid to liquid (Nie et al., 1989, 1990b). Nie et al. (1990a) also observe a band at ca. 1600 cm^{-1} for cobalamins that may correspond to the complex feature seen in Figure 4. It should be noted that band B is not observed in the cobalamin spectra of Nie et al. (1990a), pointing out the complementary nature of Raman and IR techniques. In Raman spectroscopy, the stretching modes of homopolar covalent bonds produce strong characteristic frequencies, while in IR spectroscopy the strong absorption bands come from heteropolar bonds (Parker, 1983).

In summary, we have identified the side-chain amide I mode and two corrin ring modes of cobalamins in solution. We demonstrate that these bands can be observed at concentrations reasonable for protein studies. Therefore, these results are relevant to further investigation of coenzyme/enzyme and B_{12}/transport protein interactions. For example, if the protein hydrogen-bonds to the side-chain groups of the cobalamin, we would expect the amide I frequencies of the side chains that are involved to be lowered. In addition, the modes at ca. 1575 and 1545 cm^{-1} are sensitive to changes in the structure of the corrin ring. As the frequency decreases, the corrin conjugated system expands. When cobalamins are protein-bound, the shifts in optical spectra are consistent with electron donation to the corrin ligand. Examination of the frequencies of bands C and D for protein-bound cobalamins will reveal the direct structural influences of protein binding. Changes in the C–N stretch at ca. 2130 cm^{-1} will also provide information on the effects of protein binding on the axial ligands.

Registry No. Cob(II)alamin, 14463-33-3; methylcobalamin, 13422-55-4; adenosylcobalamin, 13870-90-1; cyanocobalamin, 68-19-9; aquocobalamin, 13422-52-1; dicyanocobinamide, 27792-36-5; glutamine, 56-85-9; hydroxocobalamin, 13422-51-0.

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Spectroscopic Studies of Myoglobin at Low pH: Heme Structure and Ligation†

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ABSTRACT: We explore heme structure and ligation subsequent to a low-pH conformational transition in sperm whale myoglobin. Below pH 4.0, the iron–histidine bond breaks in metMb and deoxyMb. In MbCO, the majority of the iron–histidine bonds remain intact down to pH 2.6; however, the observation of a weak Fe–CO mode at 526 cm^{-1} indicates that a small fraction of the sample has the histidine replaced by a weak ligand, possibly water. The existence of a sterically hindered CO subpopulation in MbCO and the continued association of the four-coordinate heme with the protein in deoxyMb suggest that the heme pocket remains at least partially intact in the acid-induced conformation. The global pH-dependent conformational change described here is clearly distinguished from the local "closed" to "open" transition described previously in MbCO [Morikis et al. (1989) *Biochemistry 28*, 4791–4800]. Further observations of the four-coordinate heme state yield insights on the mechanism of heme photoreduction and the assignment of the 760-nm band in deoxyMb.

Studies of ligand binding to heme proteins are complicated by a variety of factors. For example, we have recently put forward a simple model for ligand binding in heme proteins that formally separates the proximal and distal interactions contributing to the energy barrier for binding at the heme site (Šrajer et al., 1988). The proximal interactions appear to be well explained by use of a simple harmonic approximation to the coordinate describing the iron–porphyrin out-of-plane displacement. Within this model, the distributed low-temperature geminate recombination kinetics of myoglobin (Mb)[1] (Austin et al., 1975), as well as the average heme binding activation energy at physiological temperature, are derived by using heme parameters determined from independent experimental and theoretical studies.

The forces describing the interaction of the ligand with the distal pocket are much more poorly understood. Recent investigations of Mb and hemoglobin (Hb) that utilize the power of site-directed mutagenesis (Morikis et al., 1989; Braunstein et al., 1988; Olson et al., 1989; Nagai et al., 1987) demonstrate, as expected, that the distal histidine plays a significant role in the ligand binding reaction. In fact, it appears that, even at intermediate values of pH (4–7), the distal histidine is coupled to an important protein conformational equilibrium that favors a more "open" and accessible distal pocket structure as the pH is lowered (Morikis et al., 1989). The existence of the open structure has been suggested on the basis of NMR (Lecomte & La Mar, 1985) and X-ray diffraction (Ringe et al., 1984) studies, as well as energetics calculations based on the X-ray structure (Kuriyan et al., 1986).

In the course of our studies, we have become aware of numerous investigations at low pH (2–4) that involve ligand binding to heme model systems (Geibel et al., 1975; Cannon et al., 1976), myoglobin (Giacometti et al., 1977, 1981; Coletta et al., 1985; Traylor et al., 1983; Rousseau et al., 1989; Han et al., 1990) and hemoglobin (Coletta et al., 1988). These investigations are potentially affected by both the proximal and distal interactions discussed above. Proximal interactions may be affected by the alteration of the iron–porphyrin equilibrium displacement that results from loss of the iron–histidine bond (Traylor et al., 1983). Direct resonance Raman evidence that such four-coordinate heme states are formed in

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[1] Abbreviations: Mb, sperm whale myoglobin; Hb, hemoglobin; metMb, metmyoglobin; deoxyMb, deoxymyoglobin; MbCO, carboxymyoglobin; RR, resonance Raman; CD, circular dichroism; N, native conformation; U, acid conformation, ANS, 1-anilino-8-naphthalenesulfonate.

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