Kinetic Evidence for Unexpectedly Strong Precursor Formation in Ligand Substitution Reactions of Aquacobalamin (Vitamin B_{12a})

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A detailed kinetic study of the substitution of aquacobalamin by a variety of substituted pyridine ligands was performed as function of entering ligand concentration, pH, temperature and pressure. The kinetic data for the complex formation and reverse aquation reactions confirm previous findings on the operation of a dissociative interchange mechanism. Typical $\Delta V^{\ddagger}$ values for complex formation are between +4 and +7 cm^3 mol^{-1}. The thermodynamic data reflect significant precursor formation for this special class of ligands investigated, with formation constants that vary between 1 and 5 M^{-1}. This is ascribed to an interaction of the π-system on the pyridine ligands with either the corrin moiety or other functional groups on the cobalamin molecule. Activation and thermodynamic parameters for all reaction steps involved are reported and discussed with reference to available data from the literature.

Introduction

Ligand substitution reactions of aquacobalamin (vitamin B_{12a}) are in general characterized by a linear dependence of the observed pseudo-first-order rate constant on the concentration of the entering nucleophile.[2-6] In terms of the suggested dissociative interchange (I_d) mechanism, this means that precursor formation is weak and does not show up in the kinetic data even at high entering ligand concentration. The first exception to this general behavior was found for the reaction with pyridine,[7] for which plots of k_{obs} versus pyridine concentration deviated significantly from the expected linear behavior. This was incorrectly considered as evidence for the operation of a limiting dissociative (D) mechanism, and later work[8] clearly demonstrated that the observed deviation must be due to a precursor formation step in terms of the I_d mechanism. Marques and co-workers,[9] reproduced our finding for the reaction with pyridine and found a similar behavior for 4-methylpyridine. Some deviation from linearity in the k_{obs} versus nucleophile concentration plots were also found for hydroxylamine, methyl glycinate, imidazole, and histamine, but by far not as significant as for pyridine.[9] Surprisingly, anionic nucleophiles did not exhibit this phenomenon[5,10-12] as would be expected for precursor ion-pair formation since aquacobalamin is a cation. Thus no reasonable explanation for the observed effects could be offered and a possible interaction of the pyridine ligand with the B_{12} molecule involving its π-character was suggested.[8]

We have now undertaken a more detailed study of the kinetic evidence for precursor formation in ligand substitution reactions of aquacobalamin. We have found a number of substituted pyridine ligands that exhibit a similar behavior and have determined rate and thermodynamic parameters for all the reaction steps involved in the substitution process. These data allow a detailed comparison and enable us to comment on the nature of the precursor formation process.

Experimental Section

Crystalline hydroxocobalamin hydrochloride (Fluka) was used to prepare the solutions of aquacobalamin. All solutions were prepared from Millipore water and stored in the dark at ca. 5 °C. 4-Methylpyridine, 2,4-dimethylpyridine, and 4-acetylpyridine were obtained from Fluka and 3-acetylpyridine, 1,2,4-triazole, and pyrazole from Aldrich. 2,4-Dimethylpyridine and 4-methylpyridine were distilled under vacuum before use. All other chemicals were of either analytical or reagent grade.

All reactions were followed by characteristic absorbance changes on a Durrum D110 stopped-flow instrument. At high pressure (≤150 MPa) a homemade stopped-flow instrument described elsewhere[13] was used. The UV-vis spectra were recorded on Cary 1 and on HP 8452A spectrophotometers. The rapid-scan measurements were performed with an optical simultaneous multichannel analysis (OSMA) instrument, supplied by Spectroscopy Instruments, and connected to a similar stopped-flow instrument that was used for the kinetic measurements.[14] The instruments were thermostated to within ±0.1 °C.

For pH <7.5, a ligand solution was mixed with an aquacobalamin solution of the same pH and ionic strength. A tris(hydroxymethyl)-aminomethane buffer (0.1 M) was used to stabilize the pH at higher values. Blank experiments were performed to establish that the selected buffer does not react with aquacobalamin. In all cases the ligand concentration was at least 10 times higher than the complex concentration to ensure pseudo-first-order conditions. All absorbance versus time traces were recorded and analyzed by using an IBM compatible computer with Biologic V3.23 and OLIS software. All reported k_{obs} values are pseudo-first-order rate constants. The quoted error limits for k_{obs} are the standard deviations from at least five kinetic runs.

Results and Discussion

The reaction of aquacobalamin was investigated for a variety of substituted pyridines in order to vary the basicity and donor/acceptor properties of the entering nucleophile. Some could only be studied over a very limited concentration range due to solubility limitations, whereas others showed no effective substitution reaction at all. Rate data for the more promising ligands are

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Table 1. Summary of Available Kinetic Data for the Substitution of B_{12}–H_2O^+ by Several Ligands for the Overall Reaction at 25 °C

$$B_{12}\text{–}H_2O^+ + L \rightleftharpoons B_{12}\text{–}L^+ + H_2O \quad k_a, k_b$$

L	k_a ($=k_4K_3$), M^{-1} s^{-1}	ΔH_a^*, kJ mol^{-1}	ΔS_a^*, J K^{-1} mol^{-1}	$\Delta V^*(k_a)$, cm^3 mol^{-1}	k_b ($=k_{-4}$), s^{-1}	ΔH_b^*, kJ mol^{-1}	ΔS_b^*, J K^{-1} mol^{-1}	$\Delta V^*(k_b)$, cm^3 mol^{-1}	$\Delta\bar{V}$,[a] cm^3 mol^{-1}	ref
PY	34.2 ± 0.9	73 ± 5	29 ± 17	8.7 ± 1.4	2.28 ± 0.06	86 ± 3	50 ± 10	16.9 ± 0.8	−8.2 ± 2.2	7
TU	223	69 ± 2	32 ± 6	3.6 ± 0.5	15.2	85 ± 3	61 ± 11	6.3 ± 2.5	−2.7 ± 3.0	10
	184 ± 5	63 ± 4	9 ± 15	9.1 ± 0.9	15.5 ± 0.4	83 ± 1	55 ± 5	6.7 ± 0.3	2.4 ± 1.2	8
DMTU	115 ± 2	78 ± 6	55 ± 20	6.7 ± 1.0	37.9 ± 0.2	65 ± 1	4 ± 5	10.0 ± 0.8	−3.3 ± 1.8	8
SCN^-	2.3×10^3	72 ± 2	59 ± 4		1.8	72 ± 2	2 ± 8			11
I^-	1.4×10^3	67.0 ± 0.4	20 ± 1	5.5 ± 0.8	35 ± 4			11.5 ± 1.6	−5.8 ± 2.3	11,12
$S_2O_3^{2-}$	200 ± 20	63 ± 3	8 ± 8	6.0 ± 0.8	3.5×10^{-2}	80 ± 2	−3 ± 8	−2.7 ± 0.5[c]		10,11
SO_3^{2-}	3.3 ± 0.1	79.9 ± 0.5	33 ± 1		4×10^{-3}	96 ± 7	32 ± 22			3
4-MePY	67 ± 2	66 ± 1	10 ± 4	8.2 ± 0.5						b
3-AcPY	31 ± 2	96 ± 12	105 ± 40	9.7 ± 1.1	2.61 ± 0.09	78 ± 7	26 ± 22	12.2 ± 0.5		b
4-AcPY	35 ± 1				2.34 ± 0.07					b
N_3^-	525 ± 14	65 ± 1	26 ± 2	6.4 ± 0.1						5
HN_3	103 ± 13	65 ± 3	13 ± 8	5.5 ± 0.3						5

L	k_4, s^{-1}	ΔH^*, kJ mol^{-1}	ΔS^*, J K^{-1} mol^{-1}	$\Delta V^*(k_4)$, cm^3 mol^{-1}	K_3, M^{-1}	ΔH^0, kJ mol^{-1}	ΔS^0, J K^{-1} mol^{-1}	$\Delta\bar{V}(K_3)$, cm^3 mol^{-1}	ref
PY	34 ± 7	84 ± 3	68 ± 10	7.1 ± 1.0	1.1 ± 0.2	−15 ± 7	−297 ± 24	0.8 ± 1.6	7
4-MePY	26.8 ± 0.8	61 ± 3	−14 ± 10	6.5 ± 0.5	2.5 ± 0.1	5 ± 4	24 ± 13	1.7 ± 0.7	b
3-AcPY	26 ± 2	68 ± 5	8 ± 15	3.8 ± 0.5	1.2 ± 0.1	28 ± 16	96 ± 53	5.7 ± 1.3	b
4-AcPY	20.6 ± 0.6				1.7 ± 0.1				b
2,4-DMPY	0.13 ± 0.00				4.9 ± 1.0				b

[a] $\Delta\bar{V} = \Delta V^*(k_a) - \Delta V^*(k_b)$. [b] This work. [c] In a 30% acetonitrile–water mixture.

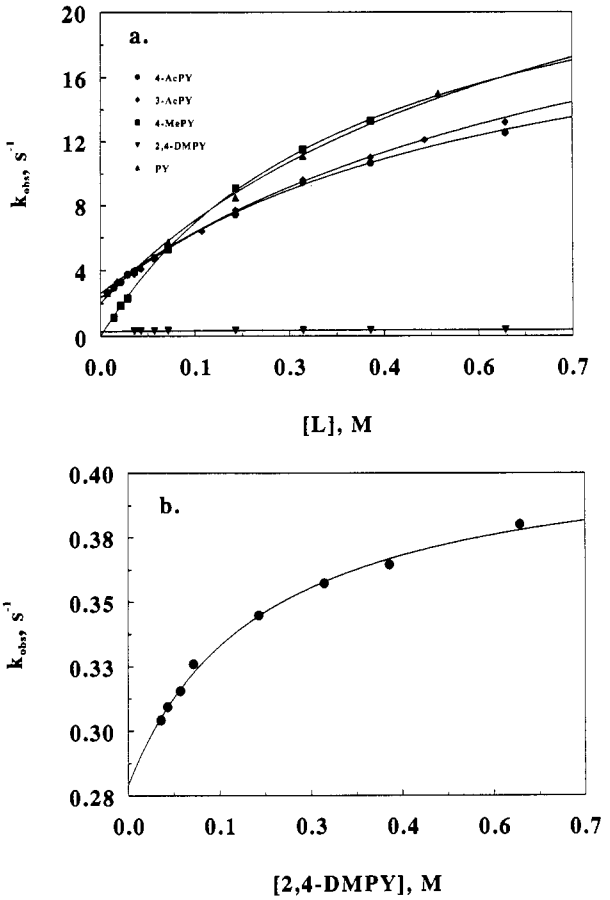

Figure 1. Dependence of k_{obs} on nucleophile concentration for substitution reactions of aquacobalamin by a series of substituted pyridines. Experimental conditions: [Co] = 7.5×10^{-5} M; 5 < pH < 7.5; ionic strength = 1.0 M (for 4-MePY 1.5 M); temperature = 25 °C.

summarized in Table 1 and Figure 1, and compared to our earlier data for the reaction with pyridine (PY).[7] Our results for the reaction with 4-methylpyridine exhibit the same trend but differ considerably from that found by Marques *et al.*[9] The difference is due to the salt selected to adjust the ionic strength. In this work $NaClO_4$ was used, whereas Marques *et al.* used KCl. We could reproduce their data by working in 1.0 M KCl and found a limiting rate constant at high 4-MePY concentration a factor 3 times lower as in $NaClO_4$ medium. This is probably due to the partial substitution of the aqua ligand by chloride in KCl medium, thus decreasing the fraction of the reactive aqua complex in solution. All kinetic measurements were performed at λ = 342 nm, where the substitution reaction is accompanied by an absorbance decrease. Kinetic measurements at 370 nm where the reaction exhibits a strong increase in absorbance, indicated the occurrence of two subsequent reaction steps, similar to that reported in the literature.[9] However, we could clearly demonstrate that this was a result of an instrumental cutoff effect at high absorbances. All subsequent experiments in the case of 4-MePY were performed at 342 nm, in order not to exceed a final absorbance higher than 1.2, and good single exponential behavior was observed.

As we have pointed out in the introduction, the nonlinear concentration dependence observed for the investigated substitution reactions in Figure 1 is unusual. We have also studied the reaction of aquacobalamine with the five-membered heterocycles 1,2,4-triazole and pyrazole (Figure 2) and could not observe a similar behavior as in the case for the pyridine ligands. The overall second-order rate constants for these ligands (Figure 2) are significantly smaller than observed for thiourea and dimethylthiourea as nucleophiles[8] and of similar magnitude as reported for pyridine as nucleophile.[7]

The observed curvature in Figure 1 can either be due to a mechanism that involves a preassociation step that results in a saturation of the k_{obs} value or due to a changeover in rate-determining step on increasing the nucleophile concentration. The latter possibility is outlined in (1), where $k_{obs} = k_1[L]$ under conditions where $k_2 \gg k_1[L]$, but becomes $k_{obs} = k_2$, i.e. independent of [L], under conditions where $k_1[L] \gg k_2$. This

$$B_{12}\text{–}H_2O^+ + L \rightarrow \text{intermediate} \quad k_1$$

$$\text{intermediate} \rightarrow B_{12}\text{–}L^+ + H_2O \quad k_2 \qquad (1)$$

changeover is merely controlled by the concentration of L and can result in the nonlinear dependence observed in Figure 1. However, this would imply that at high [L] a different reaction step is observed than at low [L], with its own characteristic spectral

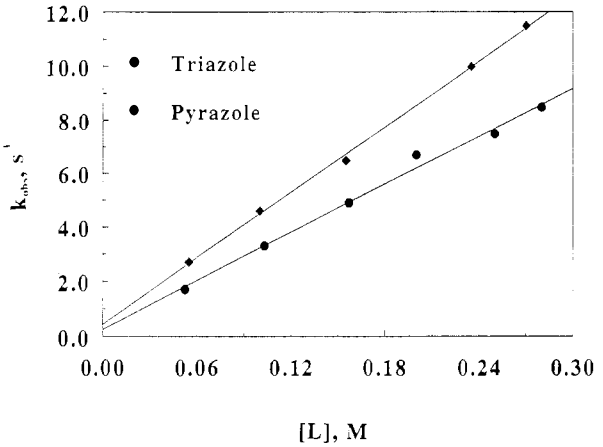

Figure 2. Dependence of k_{obs} on nucleophile concentration for substitution reactions of aquacobalamin by 1,2,4-triazole and pyrazole. Experimental conditions: [Co] = 7.5×10^{-5} M; pH = 5.0; ionic strength = 0.2 M; temperature = 25 °C.

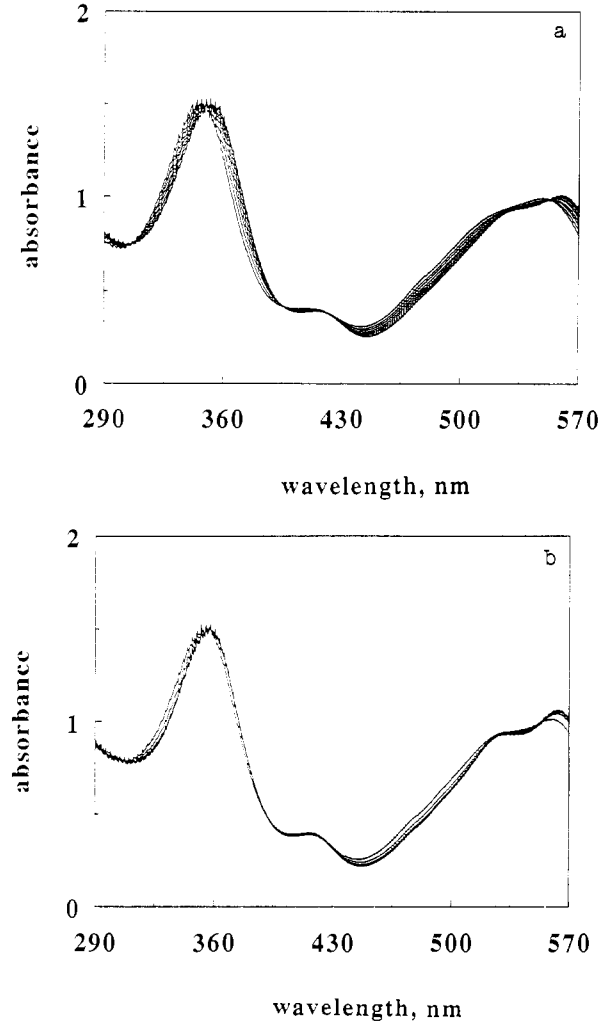

Figure 3. Rapid scan spectra recorded for the substitution of aquacobalamin by 4-MePY. Experimental conditions: [Co] = 7.5×10^{-5} M; pH = 7.0; ionic strength = 1.5 M; temperature = 25 °C; [4-MePY] = 0.02(a), 0.8(b) M; Δt = 32ms.

changes. A series of repetitive scan experiments were performed for the reaction of B_{12}–H_2O^+ with 4-MePY at nucleophile concentrations corresponding to the initial linear and final independent concentration range. The results in Figure 3 clearly demonstrate that identical spectral changes and isosbestic points are observed under all conditions, indicating that a changeover in rate-determining step does not occur.

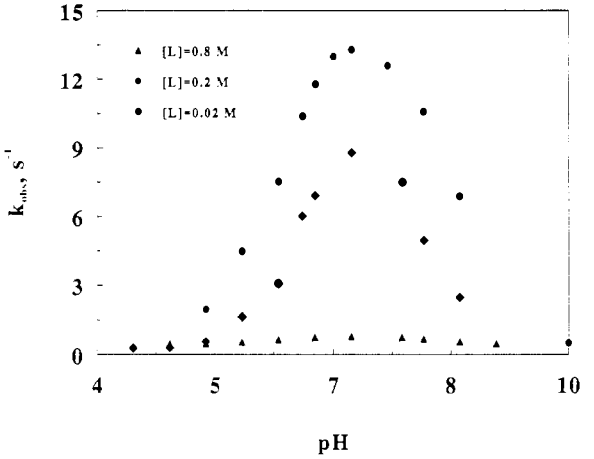

Figure 4. Dependence of k_{obs} on pH for the substitution of aquacobalamin by 4-MePY. Experimental conditions: [Co] = 7.5×10^{-5} M; ionic strength = 1.5 M; temperature = 25 °C.

Another aspect to take into consideration and that may lead to deviations in the nucleophile concentration dependence of the data, concerns the pH of the solution. Both the aquacobalamin and pyridine ligands exhibit characteristic acid-dissociation constants which result in a characteristic pH dependence of the substitution reaction.[3-5,7-9] When the mentioned pK_a values are separated by a few units from each other, a pH independent region exists where the reaction of B_{12}–H_2O^+ with the deprotonated pyridine ligand is rate-determining. A systematic variation of the pH for all studied reactions confirmed that the data reported in Table 1 and Figure 1, and subsequently in this paper, are all recorded in the pH independent region, which also was proven to be valid at low and high nucleophile concentrations (see Figure 4). It follows that the results in Figure 1 can only be interpreted in terms of the suggested and generally accepted I_d mechanism outlined in (2),[8,9] which in the present case involves a rapid precursor formation step and a rate-determining, reversible interchange step. The corresponding expression for k_{obs} is given in (3). Depending on the magnitude of the precursor formation

$$B_{12}\text{–}H_2O^+ + L \rightleftharpoons [B_{12}\text{–}H_2O^+\cdot L] \quad K_3$$

$$[B_{12}\text{–}H_2O^+\cdot L] \rightleftharpoons B_{12}\text{–}L^+ + H_2O \quad k_4, k_{-4} \qquad (2)$$

$$k_{obs} = k_{-4} + \frac{k_4 K_3 [L]}{1 + K_3 [L]} \qquad (3)$$

constant K_3, which may differ significantly for various L, plots of k_{obs} versus [L] will vary from linear to strongly curved as reported in Figure 1. The experimental data was fitted to (3) using a nonlinear least-squares fitting routine and the resulting values for K_3, k_4, and k_{-4} are summarized in Table 1. The reactions with 4-MePY and 3-AcPY were studied in detail as a function of temperature and pressure (see supplementary material), for which a typical set of data are reported in Figure 5. It follows that the non-linear concentration dependence is observed under all experimental conditions, which enabled the determination of all the activation and thermodynamic parameters included in Table 1.

From a comparison of the data in Figure 1 and Table 1, it follows that only the reaction with 4-MePY goes to completion, whereas PY, 3-AcPY and 4-AcPY go to equilibrium. In these cases the overall equilibrium constant given by k_4K_3/k_{-4} can be compared to that determined directly from the observed spectral changes.[7,8] In the present study we found a value of 9.4 ± 0.7 M^{-1} at 25 °C for the reaction with 3-AcPY, which is in good agreement with the kinetically determined value of 12 ± 1 M^{-1} under similar conditions. This formation constant is very similar

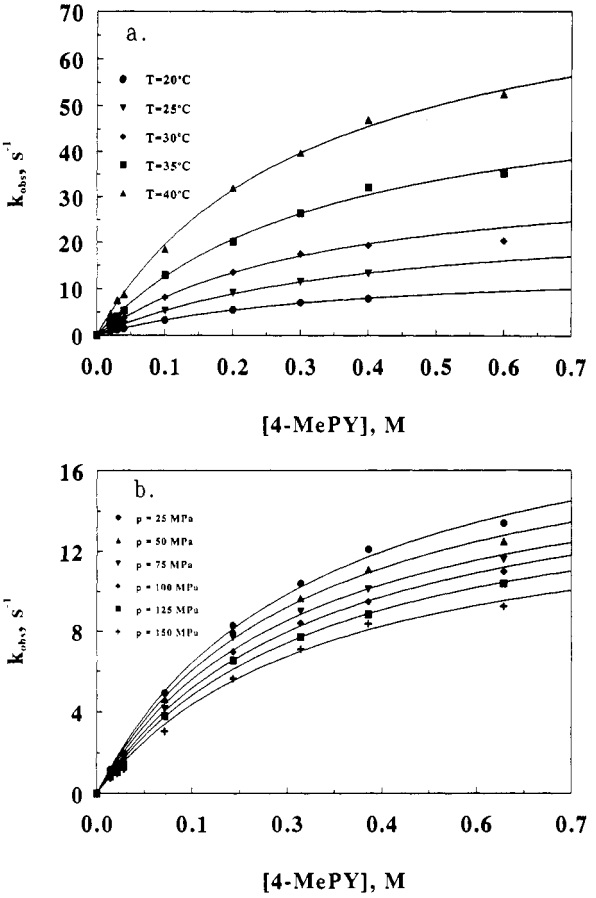

Figure 5. Dependence of k_{obs} on temperature (a) and pressure (b) for the substitution of aquacobalamin by 4-MePY. Experimental conditions: [Co] = 7.5×10^{-5} M; pH = 7.0; ionic strength = 1.5 M; temperature = 25 °C (b); pressure = 0.1 MPa (a).

to that found for the reaction with PY (18 ± 2 M^{-1}),[7] 4-AcPY (15 ± 1 M^{-1}), thiourea (18.1 ± 0.4 M^{-1}),[8] and dimethylthiourea (4.6 ± 0.2 M^{-1}).[8] Furthermore, it can be seen from the data that the aquation rate constant k_{-4} is very similar for PY (2.28), 3-AcPY (2.61), and 4-AcPY (2.34 s^{-1} at 25 °C), indicating that the cobalt–ligand bond strength must be very similar in these cases in terms of the suggested I_d mechanism. In contrast, 4-MePY must be significantly more strongly bonded to the metal center since the aquation rate constant is practically zero under these conditions. This correlates nicely with the proton basicity of the ligands, for which the pK_a values are 3.2 (3-AcPY), 3.5 (4-AcPY), 5.2 (PY), and 6.0 (4-MePY). Thus the inductive effect of the 4-Me substituent increases the electron density on the N donor atom and strengthens the Co–N bond. A larger effect is even expected for 2,4-DMPY since it has a pK_a value of 6.7, but here obviously the steric hindrance in the 2-position prevents effective complex formation (see Figure 1b).

The overall rate constants for the complex formation reaction (k_4K_3) are rather similar for the series of pyridines investigated and show no correlation with their pK_a values, which is in good agreement with that expected for an I_d mechanism that is controlled by the breakage of the Co–H_2O bond. These ligands all result in a limiting k_{obs} value at high ligand concentrations, from which a k_4 value ranging from 21 to 34 s^{-1} at 25 °C could be estimated (see Table 1). Once again the basicity of the entering nucleophile does not have a marked effect on k_4 since it is controlled by the dissociation of the coordinated water molecule. The $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ values reported for complex formation by the series of pyridines and the reverse aquation reactions in Table 1 are all significantly positive and in line with a dissociatively activated substitution mechanism. The values of $\Delta V^{\ddagger}$ for k_4 range between +3.8 and +8.7 cm^3 mol^{-1} and are very typical for solvent exchange

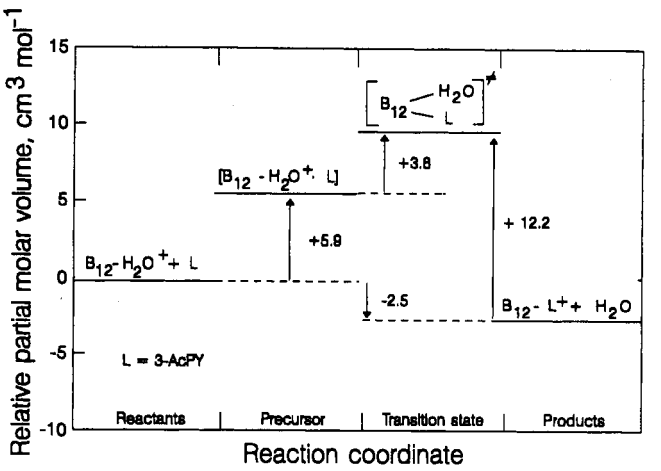

Figure 6. Volume profile for the overall reaction B_{12}–H_2O^+ + L ⇌ B_{12}–L^+ + H_2O.

in terms of an I_d mechanism.[15,16] The corresponding values for the reverse aquation reaction are somewhat larger, in agreement with the dissociation of a significantly larger molecule than for the forward reaction.

The most remarkable aspect of the investigated reactions is the significant precursor formation observed for PY, 4-MePY, 3-AcPY, 4-AcPY, and 2,4-DMPY as reflected by the values of K_3 ranging between 1.1 and 4.9 M^{-1} at 25 °C (see Table 1). These values are rather large for the association of B_{12}–H_2O^+ with a neutral ligand and, even more surprising, are much larger than that observed for the association with anionic ligands where significant ion-pair formation could contribute to the value of K_3. Furthermore, the values of K_3 seem to correlate with the pK_a values mentioned above, i.e. the basicity of the pyridines, since the largest K_3 values found for 4-MePY and 2,4-DMPY correspond to pK_a values of 6.0 and 6.7, respectively. It follows that there must be a very specific interaction of the pyridine ligands with the aquacobalamin species in order to account for these large K_3 values. By way of comparison no significant K_3 value could be detected for the other neutral and anionic ligands investigated.[2-12] The pressure dependence of K_3 results in $\Delta\bar{V}$ values ranging between 0 and +6 cm^3 mol^{-1}, indicating that precursor formation does not involve a significant change in partial molar volume of the separated species. This would mean that any volume collapse resulting from the association of the reactant molecules is offset by a volume increase resulting from desolvation of the reactants on forming a contact pair. This strong precursor formation can only be related to an interaction of the π-system on the pyridine ligands with the corrin moiety or other functional groups on the cobalamin molecule. The introduction of methyl substituents on PY increases K_3 from 1.1 to 2.5 and 4.9 M^{-1} for 4-MePY and 2,4-DMPY, respectively, indicating that an increased electron density on the π-system increases precursor formation. It is generally known from the literature that a system must be sufficiently electron rich before bonding through the π-electrons of pyridine can take place.[17] This π-interaction results in the effective binding of the nucleophile close to the metal center during precursor formation, which is followed by an interchange of ligands controlled by the breakage of the Co–H_2O bond in terms of an I_d mechanism.

The results reported in this study can be used to construct a volume profile for the reaction with 3-AcPY. Such profiles have been shown to be very useful in describing the intimate nature of substitution processes.[16] Figure 6 clearly demonstrates the dissociative nature of the ligand substitution process, where the

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transition state has a significantly higher partial molar volume than either the reactant and product states. The overall substitution reaction is characterized by a volume decrease resulting from the substitution of a small water molecule by a significantly larger pyridine molecule. This volume profile is in close agreement with that reported for the reaction with pyridine, the main difference being significantly smaller $\Delta\bar{V}(K_3)$ and larger $\Delta V^{\ddagger}(k_b)$ values in the latter case.[8] The values of $\Delta V^{\ddagger}(k_a)$ for the overall complex formation reaction, viz. 8.7 ± 1.4 and 9.7 ± 1.1 $cm^3\,mol^{-1}$ for PY and 3-AcPY, respectively, are remarkably close and illustrate the I_d nature of the process. Thus the smaller $\Delta\bar{V}(K_3)$ value is accompanied by a larger $\Delta V^{\ddagger}(k_4)$ value in the case of PY and vice versa in the case of 3-AcPY. Thus precursor formation and the subsequent interchange reaction step are strongly interlinked processes in terms of the associated volume changes, which further supports our suggestion of efficient precursor binding close to the metal center for these type of ligands.

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Supplementary Material Available: Tables of k_{obs} data for the reaction of B_{12}–H_2O^+ with 4-MePY, 3-AcPY, 4-AcPY, and 2,4-DMPY as a function of nucleophile concentration, temperature, and pressure (5 pages). Ordering information is given on any current masthead page.