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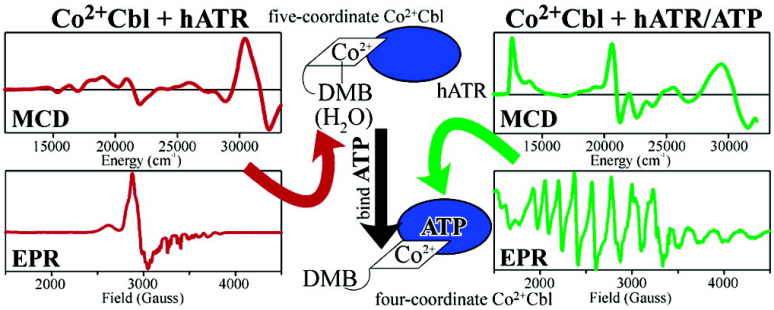


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# Spectroscopic Evidence for the Formation of a Four-Coordinate $Co^{2+}$Cobalamin Species upon Binding to the Human ATP:Cobalamin Adenosyltransferase

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The adenosylated derivative of vitamin $B_{12}$, known as adenosylcobalamin (AdoCbl), is essential to all mammals, as it serves as the cofactor for the enzyme methylmalonyl-CoA mutase (MMCM) that detoxifies the cell of methylmalonyl-CoA, a harmful catabolite if allowed to accumulate. Humans are unable to synthesize AdoCbl de novo; rather, they assimilate exogenous cobalamin and convert it to AdoCbl using an enzyme that transfers the adenosyl group from ATP to the cobalamin cosubstrate (Figure 1). Malfunction of this human adenosyltransferase (hATR) can lead to the potentially fatal disease methylmalonic aciduria.[1,2] Hence, elucidation of the molecular mechanism of cobalamin adenosylation catalyzed by hATR is of considerable interest. Probably the most fascinating, yet still poorly understood, aspect of this process is how hATR activates the cobalamin substrate for $Co^{2+}Cbl \rightarrow Co^{1+}Cbl$ reduction, as the corresponding reduction midpoint potential of $-610$ mV versus SHE is below that of in vivo reducing agents (step iii, Figure 1).[3,4] We have recently demonstrated that the electronic structures of $Co^{2+}Cbl$ and related $Co^{2+}$corrinoids can be probed in detail using magnetic circular dichroism (MCD) spectroscopy.[5] A particularly significant finding from these studies was that the Co d $\rightarrow$ d transitions of $Co^{2+}Cbl$, which dominate the 11 000$-$21 000 $cm^{-1}$ region of the corresponding MCD spectrum (Figure 2A), shift considerably upon substitution of the dimethylbenzimidazole (DMB) lower axial ligand by a more weakly coordinating water molecule to generate the "base-off" form (Figure 2B). Building upon these insights, we have engaged in MCD and electron paramagnetic resonance (EPR) studies aimed at evaluating the effects of the $Co^{2+}Cbl$/hATR interactions in both the absence and the presence of ATP on the geometric and electronic structures of the cobalamin cofactor. Here we present spectroscopic evidence for the formation of an unprecedented four-coordinate $Co^{2+}Cbl$ intermediate during AdoCbl biosynthesis catalyzed by hATR.

The MCD spectrum of $Co^{2+}Cbl$ bound to hATR in the absence of ATP exhibits only small differences compared to that of free base-on $Co^{2+}Cbl$ (cf. Figure 2C,A). Likewise, X-band EPR data of the free (base-on) and hATR-bound $Co^{2+}Cbl$ cofactor are very similar (cf. Figure 3C,A); however, observation of the positive feature at ~2700 G and an octet of hyperfine lines split by 144 G, centered at $g = 2.0$ in the protein spectrum (Figure 3C), discloses the presence of a sizable fraction of base-off $Co^{2+}Cbl$ (Figure 3B) in this sample. Indeed, a quantitative analysis of the MCD and EPR data in Figures 2C and 3C reveals that the corresponding samples contained ~60% base-on and 40% base-off $Co^{2+}Cbl$.[6,7]

Compared to the minor MCD spectral changes accompanying binding of $Co^{2+}Cbl$ to ATP-free hATR, all of which can be rationalized in terms of a partial conversion of the cofactor to its base-off form, much more dramatic changes are observed in the presence of ATP (Figure 2D). Most noticeably, the lowest-energy transition exhibits a sizable red-shift and a massive increase in intensity, signaling the formation of a strongly perturbed $Co^{2+}Cbl$ species. Comparison of this spectrum to that in Figure 2C indicates that ~10% of the $Co^{2+}Cbl$ in the former sample was unaffected by the presence of ATP; this contribution was subtracted out to obtain the spectrum in Figure 2E. The intensities of all features in

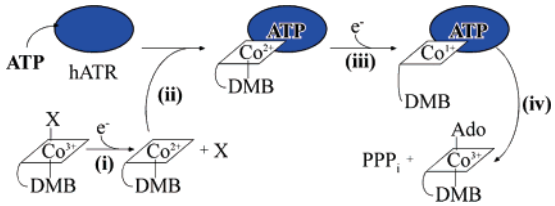


***Figure 1.*** Proposed mechanism for cobalamin adenosylation catalyzed by hATR. Assimilated cobalamin (X = $H_2O$, $CN^-$, etc.) is reduced to $Co^{2+}$-Cbl (i) that then binds to the hATR/ATP complex (ii). Further reduction yields a nucleophilic four-coordinate $Co^{1+}$ intermediate (iii) that attacks the 5′-carbon of the cosubstrate ATP to generate AdoCbl and tripolyphosphate (iv).

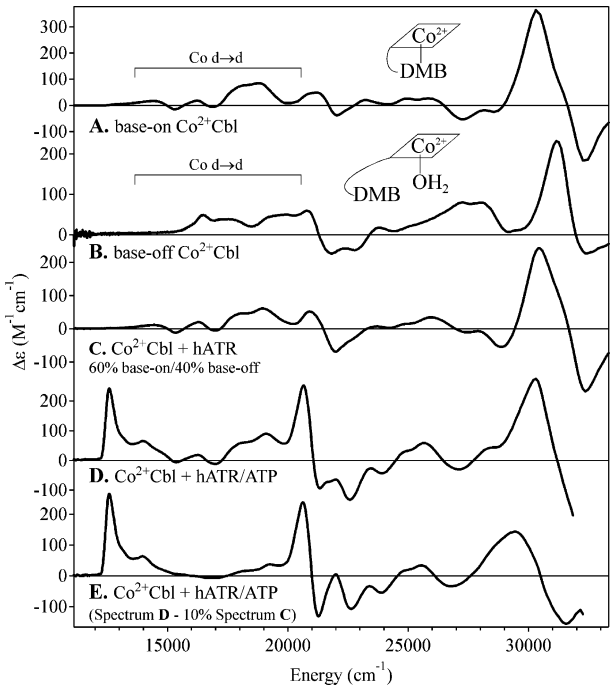


***Figure 2.*** MCD spectra at 4.5 K/7 T of (A) base-on $Co^{2+}Cbl$, (B) base-off $Co^{2+}Cbl$, (C) $Co^{2+}Cbl$ in the presence of hATR (0.25:1.0 ratio), and (D) $Co^{2+}Cbl$ in the presence of hATR (0.25:1.0 ratio) and a 20-fold excess of ATP. Spectrum E was obtained by subtracting 10% of spectrum C from spectrum D. Protein samples contained 0.45 mM hATR in 10 mM TRIS/HCl buffer (pH 8) and 60% (v/v) of the glassing agent glycerol.

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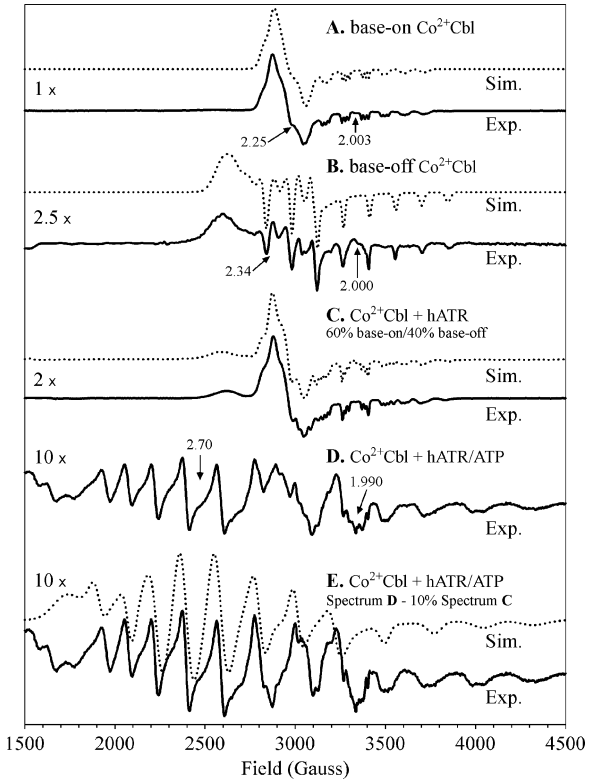


***Figure 3.*** Simulated (dotted lines) and experimental (solid lines) X-band (9.35 GHz) EPR spectra at 40 K of (A) base-on $Co^{2+}$Cbl, (B) base-off $Co^{2+}$Cbl, (C) $Co^{2+}$Cbl in the presence of hATR, and (D) $Co^{2+}$Cbl in the presence of hATR and a 20-fold excess of ATP. Spectrum E was obtained by subtracting 10% of spectrum C from spectrum D. See caption of Figure 2 for sample concentrations. Experimental conditions: modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.3 s. Spectra were scaled by the factors indicated at the left.

***Table 1.*** EPR *g*-Values and $^{59}$Co Hyperfine Values *A*(Co) (in MHz) from Spectral Simulations in Figure 3

| $Co^{2+}$Cbl | $g_1$ | $g_2$ | $g_3$ | $A_1$(Co) | $A_2$(Co) | $A_3$(Co) |
|---|---|---|---|---|---|---|
| base-on | 2.003 | 2.230 | 2.280 | 305 | 40 | 30 |
| base-off | 2.000 | 2.338 | 2.338 | 405 | 220 | 220 |
| hATR/ATP | 1.990 | 2.699 | 2.705 | 770 | 805 | 595 |

this spectrum exhibit temperature dependence consistent with an $S = 1/2$ species (Figure S1). In the low-energy region where Co d → d transitions are expected to dominate the MCD spectrum, two prominent features are observed at 13 000 and 20 000 $cm^{-1}$. Significantly, this splitting pattern of Co d → d transitions is reminiscent of four-coordinate, square-planar $Co^{2+}$ complexes.[8]

Similarly, the EPR spectrum of the $Co^{2+}$Cbl/hATR/ATP ternary complex (Figure 3D) is unlike any spectrum ever reported for a $Co^{2+}$corrinoid. Following the same procedure used to deconvolute our MCD data, contributions from the $Co^{2+}$Cbl fraction of the sample not affected by ATP were removed to yield the trace shown in Figure 3E. Relevant EPR parameters obtained from a fit of this difference spectrum are listed in Table 1 along with those determined for base-on and base-off $Co^{2+}$Cbl.[9] While the *g*-tensor of this new $Co^{2+}$Cbl species retains fairly axial symmetry, a considerable increase in $g_\perp$ (i.e., $g_2$ and $g_3$) values is observed, which can be rationalized in terms of a decrease in the $d_{xz,yz}/d_{z^2}$ orbital energy splitting resulting from a significant stabilization of the Co $3d_{z^2}$ orbital. In support of this model, the dramatic increase in $^{59}$Co hyperfine values from base-on to base-off to hATR/ATP-bound $Co^{2+}$Cbl, signifying an increase in unpaired spin density on the Co center,[5] can be attributed to a weakening of the cobalt−axial ligand bonding interaction along this series. Interestingly, the EPR parameters for $Co^{2+}$Cbl bound to the hATR/ATP complex lay between typical values for four-coordinate and five-coordinate $Co^{2+}$-octaethylporphyrins (OEP),[10] providing further evidence that this new species contains a low-spin $Co^{2+}$ center lacking any significant axial bonding interactions.

In conclusion, our MCD and EPR spectroscopic data reported here indicate that in the absence of the cosubstrate ATP, the interaction between $Co^{2+}$Cbl and hATR promotes partial conversion of the cofactor to the base-off form in which a water molecule occupies the lower axial position.[6] This enzyme-induced base-on → base-off conversion should raise the $Co^{2+/1+}$ reduction midpoint potential by 120 mV,[3] thereby activating the cobalamin substrate for reduction. The perturbations of the $Co^{2+}$Cbl geometric and electronic structures are much more pronounced in the presence of ATP, revealing the formation of a $Co^{2+}$Cbl species with unprecedented spectroscopic properties. On the basis of a comparison to published EPR parameters for $Co^{2+}$(OEP) complexes[10] and consistent with preliminary DFT and TD-DFT computations,[5] this unique species is described as possessing a low-spin $Co^{2+}$ center in an approximate four-coordinate, square-planar ligand environment (i.e., lacking any significant axial bonding interactions). Such a $Co^{2+}$ coordination geometry is expected to promote facile electron transfer into the redox-active Co $3d_{z^2}$-based molecular orbital, effectively raising the $Co^{2+/1+}$ reduction potential into the physiological range. Observation of this highly activated $Co^{2+}$ species only in the presence of ATP suggests that the enzyme exerts significant control over the timing of $Co^{2+}$Cbl → $Co^{1+}$Cbl reduction, thereby protecting itself from deleterious side reactions by the transiently formed $Co^{1+}$Cbl "supernucleophile".

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**Supporting Information Available:** Experimental details, temperature-dependent variable-field MCD data, and EPR simulation parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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