Substrate-Induced Conformational Change of a Coenzyme B_{12}-Dependent Enzyme: Crystal Structure of the Substrate-Free Form of Diol Dehydratase[†,‡]

Naoki Shibata,[§] Jun Masuda,[§] Yukio Morimoto,[§] Noritake Yasuoka,[§] and Tetsuo Toraya*[,||]

Department of Life Science, Graduate School of Science, Himeji Institute of Technology, 3-2-1 Kouto, Kamigori, Ako-gun, Hyogo 678-1297, Japan, and Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan

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ABSTRACT: Substrate binding triggers catalytic radical formation through the cobalt−carbon bond homolysis in coenzyme B_{12}-dependent enzymes. We have determined the crystal structure of the substrate-free form of *Klebsiella oxytoca* diol dehydratase·cyanocobalamin complex at 1.85 Å resolution. The structure contains two units of the heterotrimer consisting of α, β, and γ subunits. As compared with the structure of its substrate-bound form, the β subunits are tilted by ~3° and cobalamin is also tilted so that pyrrole rings A and D are significantly lifted up toward the substrate-binding site, whereas pyrrole rings B and C are only slightly lifted up. The structure revealed that the potassium ion in the substrate-binding site of the substrate-free enzyme is also heptacoordinated; that is, two oxygen atoms of two water molecules coordinate to it instead of the substrate hydroxyls. A modeling study in which the structures of both the cobalamin moiety and the adenine ring of the coenzyme were superimposed onto those of the enzyme-bound cyanocobalamin and the adenine ring-binding pocket, respectively, demonstrated that the distortions of the Co−C bond in the substrate-free form are already marked but slightly smaller than those in the substrate-bound form. It was thus strongly suggested that the Co−C bond becomes largely activated (labilized) when the coenzyme binds to the apoenzyme even in the absence of substrate and undergoes homolysis through the substrate-induced conformational changes of the enzyme. Kinetic coupling of Co−C bond homolysis with hydrogen abstraction from the substrate shifts the equilibrium to dissociation.

Adenosylcobalamin (AdoCbl)[1] (coenzyme B_{12})-dependent rearrangements (Figure 1A) are catalyzed by a radical mechanism (*1*). The catalytic radical in these reactions is an adenosyl radical that is generated from the coenzyme by homolytic cleavage of its Co−C bond [Figure 1B(i)]. The essential steps common to all the AdoCbl-dependent rearrangements are two hydrogen abstraction steps, i.e., hydrogen abstraction of the adenosyl radical from substrates and hydrogen back-abstraction (recombination) of a product-derived radical from 5′-deoxyadenosine, and a group X migration, i.e., rearrangement of a substrate radical to a product radical [Figure 1B(ii)].

How does the enzyme catalyze the homolysis of the coenzyme Co−C bond? This has been one of the most important questions that has needed to be answered. In diol dehydratase, earlier structure−function studies of AdoCbl using synthetic coenzyme analogues indicated that tight interactions of the coenzyme with the apoenzyme at both the cobalamin moiety and the adenosyl group are indispensable for activation of this organometallic bond (*2*−*7*). However, the interaction of the adenosyl group with the apoenzyme remained unclear for a long time (*8*). Recently, we reported the X-ray structures of diol dehydratase complexed with adeninylpentylcobalamin as well as with CN-Cbl at cryogenic temperatures and identified the specific adenine ring-binding site in the structure of the enzyme·adeninylpentylcobalamin complex (*9*, *10*). A modeling study revealed that tight interactions between the enzyme and coenzyme at both the cobalamin moiety and the adenine ring of the adenosyl group produce angular strains and tensile force that inevitably break the Co−C bond (*10*) (Figure 1C). Rotation of the ribosyl moiety around the glycosidic linkage makes C(5′) of the adenosyl radical accessible to the hydrogen atom to be abstracted (Figure 1D). Interactions of the enzyme with the ribose moiety of the adenosyl group as well as with the adenine ring of 5′-deoxyadenosine have been reported with methylmalonyl-CoA mutase (*11*) and glutamate mutase (*12*) as well.

It should be noted that substrate binding triggers catalytic radical formation through Co−C bond homolysis in the AdoCbl-dependent enzymes. In the holoenzyme of diol dehydratase, only a small fraction of the enzyme-bound coenzyme is in the dissociated form in the absence of substrate (*2*, *13*, *14*). When the substrate is added to the system, most of the coenzyme undergoes homolysis of its Co−C bond at a kinetically competent rate (*15*). This may

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* To whom correspondence should be addressed. Fax: 81-86-251-8264. E-mail: toraya@biotech.okayama-u.ac.jp.

[§] Himeji Institute of Technology.

[||] Okayama University.

[1] Abbreviations: AdoCbl, adenosylcobalamin; CN-Cbl, cyanocobalamin; LDAO, lauryl dimethylamine oxide; PEG, polyethylene glycol; KIE, kinetic isotope effect.

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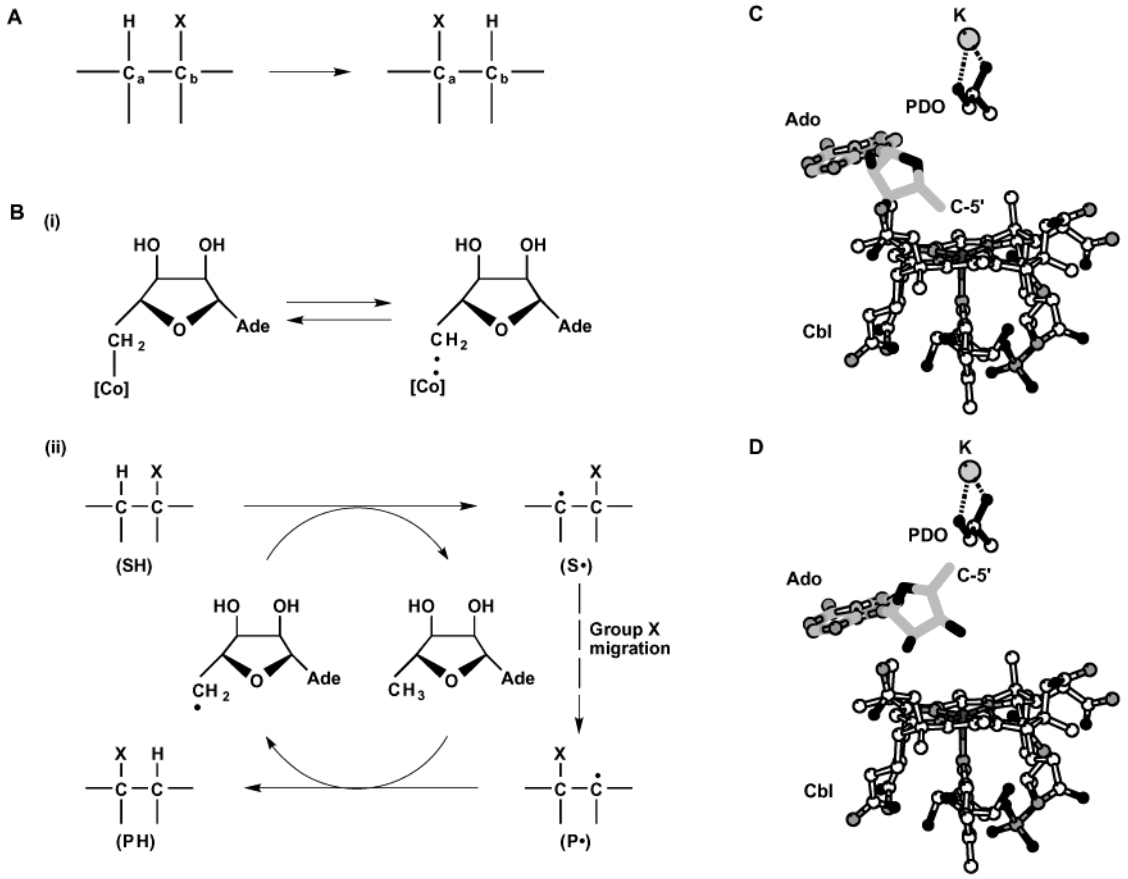

FIGURE 1: Minimal mechanism for the AdoCbl-dependent rearrangements and two conformations of the enzyme-bound adenosyl radical. (A) General representation of AdoCbl-dependent rearrangements. X is a generic migrating group. (B) Minimal mechanism for the rearrangements. (i) Homolysis of the Co−C bond of enzyme-bound AdoCbl. (ii) Adenosyl radical-catalyzed enzymatic rearrangements. [Co] is cobalamin and X a generic migrating group. Proximal (C) and distal (D) conformations of the adenosyl radical (gray sticks). The carbon, oxygen, and nitrogen atoms are represented as white, black, and light gray balls, respectively, and both the cobalt and phosphorus atoms as dark gray balls. Bonds in the substrate are shown as black sticks. Cbl is cobalamin, Ado the adenosyl group, and PDO 1,2-propanediol.

be due to a substrate-induced shift of the equilibrium and a substrate-induced conformational change of the enzyme that brings about further strains and triggers homolytic cleavage of the Co−C bond. Resonance Raman spectra showed that AdoCbl binding to methylmalonyl-CoA mutase in the absence of substrate changes the corrin ring conformation but leaves the Co−C bond unaffected (*16*). Acceleration of the rate of Co−C bond homolysis upon coenzyme binding to the apoenzyme in the presence of substrate was explained by Pratt (*17*) in terms of steric crowding at the upper face of AdoCbl.

In this paper, we report the X-ray structure of the substrate-free form of the diol dehydratase•CN-Cbl complex. By comparison of the structures of the enzyme with and without substrate, conformational changes of the enzyme upon substrate binding became obvious. These are discussed here in relation to the substrate triggering mechanism for Co−C bond homolysis. Among the AdoCbl-dependent enzyme X-ray structures that are available at present (*9*, *18*−*21*), the structure of the substrate-free form has been reported only with methylmalonyl-CoA mutase (*11*). Evans and co-workers indicated that substrate binding to methylmalonyl-CoA mutase induces a conformational change that was suggested to destroy the binding site for the adenosyl group, displacing it from the cobalt atom and forming the radical species (*11*).

EXPERIMENTAL PROCEDURES

Preparation of the Substrate-Free Diol Dehydratase•CN-Cbl Complex. Diol dehydratase from *Klebsiella oxytoca* ATCC 8724 overexpressed in *Escherichia coli* was purified following the method of Toraya and co-workers (*22*, *23*). A purified preparation of the apoenzyme containing 2.0% 1,2-propanediol was dialyzed against 20 mM potassium phosphate buffer (pH 8.0) containing 5 mM 1,2-propanediol and 0.1% LDAO. To remove the remaining 1,2-propanediol, a trace amount of AdoCbl was added to the solution so that its final molar concentration becomes 1/1000 of that of the apoenzyme, and the solution was incubated at 30 °C for 30 min. To the substrate-free apoenzyme thus obtained was added CN-Cbl to a final concentration of 2 mM. The mixture was incubated at 30 °C for an additional 30 min, followed by dialysis against 10 mM potassium phosphate buffer (pH 8.0), 0.10% LDAO, and 20 μM CN-Cbl to remove excess unbound CN-Cbl.

Crystallization. The final solution contained 50 mg/mL enzyme•CN-Cbl complex, 10 mM potassium phosphate buffer (pH 8.0), 0.10% LDAO, and 20 μM CN-Cbl. Crystals were grown by sandwich-drop vapor diffusion at 4 °C against a reservoir containing 15% (w/v) PEG6000, 0.24 M lithium chloride, and 20 mM Tris•HCl buffer (pH 8.0). The crystals were transferred to a cryosolvent containing 10 mM potas-

Table 1: Crystallographic Statistics

data collection	
wavelength (Å)	0.708
resolution limit (Å)	1.85
space group	$P2_12_12_1$
unit cell (Å)	
a	75.79
b	122.40
c	207.59
no. of measured reflections	1656266
no. of unique reflections	126694
R_{merge}[a]	0.054 (0.176)
completeness[a] (%)	76.7 (53.6)
refinement	
resolution range (Å)	10.0−1.85
R_{work}/R_{free}	0.181/0.256
total no. of atoms	14453

[a] The values in parentheses are for the highest-resolution shell (1.92−1.85 Å).

sium phosphate buffer (pH 8.0), 20 mM Tris·HCl buffer (pH 8.0), 30% sucrose, 18% PEG20000, 0.45 M potassium chloride, 0.10% LDAO, and 20 μM CN-Cbl.

X-ray Analysis and Refinement. The data sets were measured at the BL41XU beam line, SPring-8, Japan, at 100 K after the crystals had been flash-cooled. Two crystals yielded sharp diffraction spots, and two data sets were collected from them. The higher-resolution data set was used for determination of the structure presented here. The other data set contains lower-resolution data (2.0 Å) due to irradiation damage, but its completeness is higher (92.8%) even in the highest-resolution shell (80.7% for 2.00−2.07 Å). No irradiation damage was seen on the higher-resolution data, although the completeness is lower (Table 1) probably due to a shorter exposure time. The crystal belongs to space group $P2_12_12_1$ ($a = 75.79$ Å, $b = 122.40$ Å, and $c = 207.59$ Å) with an $(\alpha\beta\gamma)_2$ arrangement in the asymmetric unit. The structure of substrate-bound diol dehydratase at 100 K (PDB entry 1EGM) (*10*) was used as an initial model. Before the refinement was started, the coordinates of the substrate molecules were deleted from the original PDB data. The initial models were subjected to rigid-body refinement before following refinement and rebuilding by using CNS (*24*) and XFIT (*25*). After the first refinement step, σ_A-weighted $2F_o - F_c$ and $F_o - F_c$ maps showed some electron densities at the substrate binding sites, but they did not indicate the structure of any kinds of compounds that can be the substrate. Those electron densities were assigned to water molecules as judged by the shape of the electron densities. From the next step, the models were refined with SHELX (*26*) for the last 10 cycles.

The same refinement procedure was followed with the lower-resolution data set. The refined model was quite similar to that obtained from the higher-resolution data set. The Co−C distance (3.0 Å) obtained by the following modeling study also coincided very well with the value obtained from the higher-resolution data set. These lines of evidence indicate the reliability of the structure reported here.

Stereochemical parameters of cobalamin were calculated with GEOMCALC in the CCP4 suite (*27*). Unless otherwise stated, structural figures were created with MOLSCRIPT (*28*) and RASTER3D (*29*).

Energy Minimization for a Modeling Study. Structural information regarding the position of the coenzyme's adenine ring is not directly available from the present structure of the substrate-free enzyme·CN-Cbl complex, but the putative adenine ring-bound position in the enzyme·CN-Cbl complex could be determined by superimposition of the present structure with that of the ternary complex between the enzyme, adeninylpentylcobalamin, and 1,2-propanediol (*10*), followed by energy minimization. The initial position of the adeninylpentyl group was obtained by least-squares minimization of the α subunits between the substrate-bound enzyme·adeninylpentylcobalamin complex and the substrate-free enzyme·CN-Cbl complex. Since van der Waals contact between the adenine ring and cobalamin was too tight [the closest distance between C(4) of the adenine ring (C4A atom) and the axial CH_3 carbon (C46 atom) on C(12) of cobalamin is 2.94 Å] in the initial structure, energy minimization was applied using the program CNS without experimental data. Prior to this calculation, the torsion angle between the CB and OG atoms of Serα224 was modified so that the N3A atom of the adenine ring and the OG atom of Serα224 form a hydrogen bond as found in the structure of the enzyme·adeninylpentylcobalamin complex (*10*). Hydrogen atoms except those bound to carbon atoms were generated (the parameter hydrogen_flag was set to true). Energy minimization was performed using the model_minimize.inp input file with the default setting. Two hundred steps of conjugate gradient minimization gave a converged model. The same superimposition and energy minimization were carried out for the substrate-bound form of the enzyme·CN-Cbl complex as well using its structure obtained at 100 K (*10*).

RESULTS AND DISCUSSION

Overall Structure. The crystal structure of substrate-free form of the diol dehydratase·CN-Cbl complex was determined at 1.85 Å resolution (Table 1). There is one dimer of the $\alpha\beta\gamma$ heterotrimer unit in a crystallographic asymmetric unit. The cobalamin molecule sits on the β subunit in the "base-on" mode, that is, with the 5,6-dimethylbenzimidazole of the nucleotide moiety coordinating to the cobalt atom (*9*), and the C-terminal side of the $(\beta/\alpha)_8$ barrel in the α subunit covers the upper side of the cobalamin. The substrate-binding site, including the catalytically essential K^+ ion (*30*, *31*), is in the $(\beta/\alpha)_8$ barrel.

The overall structure of the substrate-free form of the enzyme, including active site residues, is essentially similar to that of the substrate-bound form (*10*) with an rmsd of 0.52 Å. This value is larger by 0.08 Å than the rmsd between the $\alpha\beta\gamma$ dimers of the substrate-free form. This means that substrate binding causes distinct conformational changes of the enzyme·CN-Cbl complex. Superimposition of the structures shows that the major structural changes upon substrate binding are located in the β subunit and in the region of the α subunit that interacts directly with the β subunit (Figure 2A). Although the substrate binds to the α subunit, the conformational change in the whole α subunit is much smaller than that in the β subunit. The β subunit is tilted by ∼3° relative to its position in the substrate-bound form. The axis of rotation in the tilting is in the line passing the N atom of Glnβ195 and parallel to the line connecting the N22 (ring B) and N24 (ring D) atoms of the corrin ring. This axis vertical to the sheet is shown as the black dot in Figure 2A. The contact area between the α and β subunits was essentially not affected by the presence of substrate.

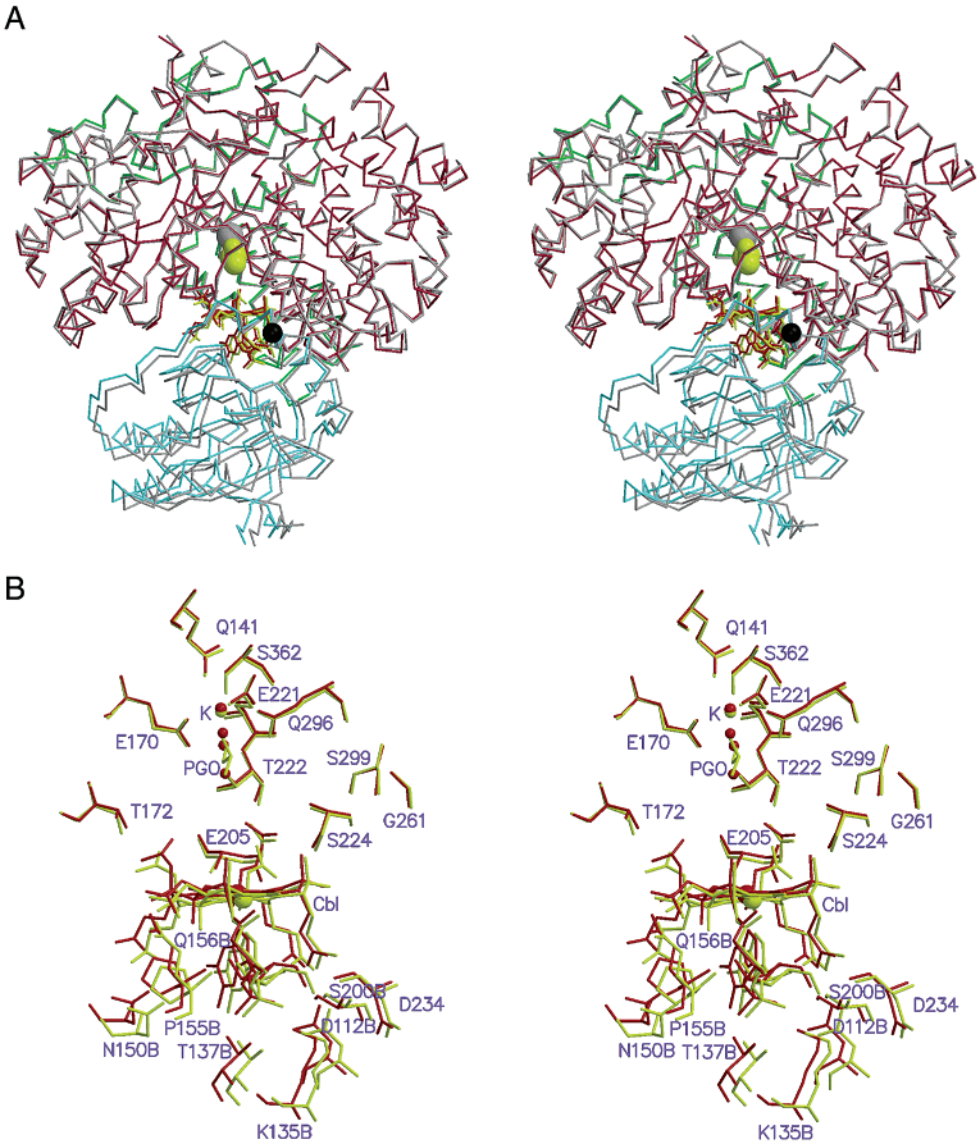

FIGURE 2: Comparison of the structures of substrate-free and substrate-bound forms of diol dehydratase. (A) Stereodrawing of the Cα superposition of the first $\alpha\beta\gamma$ unit (chains A, B, and G). The least-squares superposition minimized the rmsd of the Cα atoms only in the α subunit. In the substrate-free form, α, β, and γ subunits are colored red, cyan, and green, respectively. The Cα model of the substrate-bound form is shown in gray. Yellow and gray spheres represent the substrate and K^+ ion of the substrate-bound form, respectively. Cobalamin molecules of substrate-free and substrate-bound forms are shown in red and yellow stick representations, respectively. The axis of the domain tilt is vertical to the sheet and passes the N atom of Glnβ195 which is shown as a black dot. (B) Movement of active site residues and cofactors upon substrate binding. Red and yellow models represent substrate-free and substrate-bound forms, respectively. Cobalamin (labeled as Cbl), residues within 3.5 Å of cobalamin, 1,2-propanediol (labeled as PGO), and K^+ ion are shown. Cobalt atom, K^+ ion, and water molecules are depicted as larger spheres. The label B after the residue number refers to residues of the β subunit. For clarity, the direction of the view is slightly changed from that of panel A.

Cobalamin Binding and the Conformation of Bound Cobalamin. The cobalamin, which is bound on the interface between the α and β subunits, moves along with the movement of the β subunit so that it lengthens the Co−K^+ distance upon substrate binding, rather than staying with the α subunit (Figure 2B). Drastic hydrogen bond rearrangement does not occur because the movement is very subtle compared with that of methionine synthase recently reported (*32*) in which its corrin ring is tilted by 18°.

The Co−N bond distance between Co and N(3) of 5,6-dimethylbenzimidazole, the lower axial ligand, in the substrate-free form of the enzyme·CN-Cbl complex is 2.25 Å. This value is close to those in the substrate-bound form of the same complex (2.18 Å) and the substrate-bound form of the enzyme·adeninylpentylcobalamin complex (2.22 Å) (*10*), but significantly shorter than that in the substrate-bound enzyme·cobalamin complex (2.50 Å) (*9*). We assigned the latter to the complex of the enzyme with cob(II)alamin, because the Co−CN bond can be cleaved by X-ray irradiation during data collection with diol dehydratase (*10*, *33*). Kratky and co-workers have reported that free and glutamate mutase-bound CN-Cbl is reduced to cob(II)alamin by X-ray irradiation (*34*). The electron density corresponding to the CN group was partly observed with the diol dehydratase·CN-Cbl complex in the absence of substrate.

Superimposition of the cobalamin in the substrate-free form with that in the substrate-bound form (*10*) showed that the conformations of cobalamin in both forms are basically the same with rmsds of 0.06 Å calculated for the corrin rings and of 0.27 Å calculated for those including side

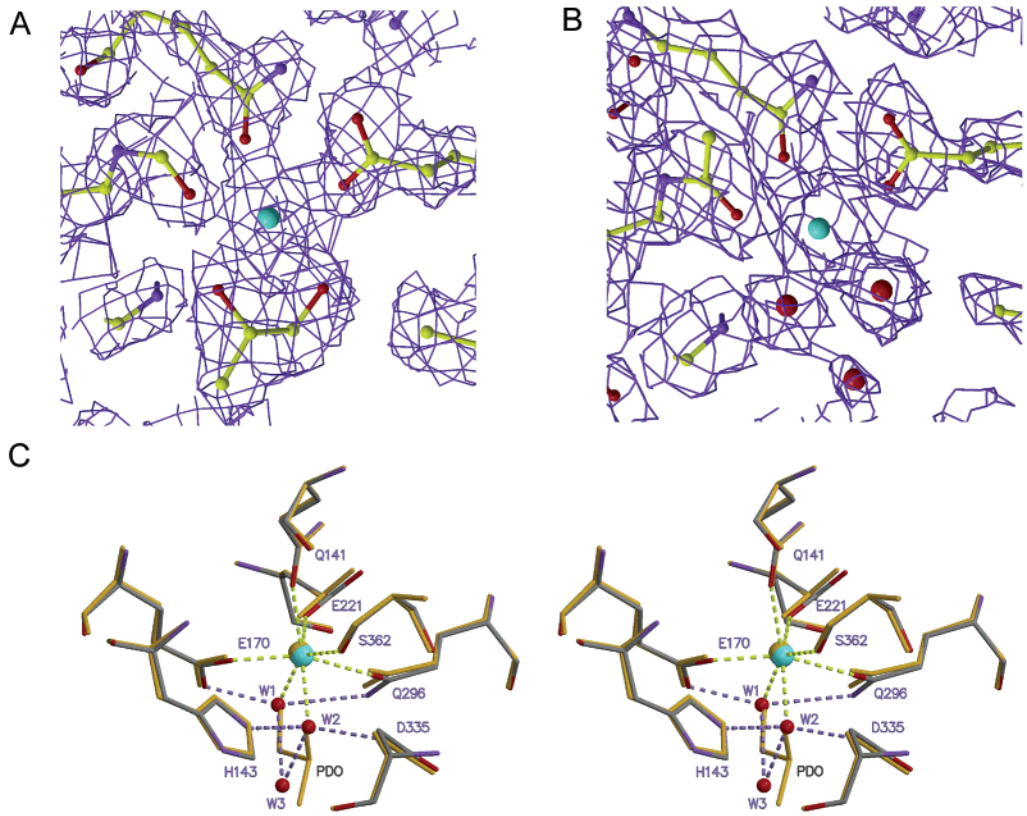

FIGURE 3: Substrate binding site. Panels A and B were created with XFIT (*25*) and RASTER3D (*29*). (A) Substrate-bound form with a $2mF_o - DF_c$ electron density map contoured at the 1.0σ level. (B) Substrate-free form with a $2mF_o - DF_c$ electron density map (1σ level). (C) Comparison of the structures between substrate-free and substrate-bound forms: gray for carbon, red for oxygen, blue for nitrogen, and cyan for K^+ ion in the substrate-free form and dark orange for all the atoms in the substrate-bound form. The yellow dotted lines represent coordination of the ligands to the cobalt atom. The blue dotted lines represent hydrogen bonds.

chains. However, it should be noted that pyrrole rings A and D in the substrate-free form are significantly lifted up toward the substrate-binding site, whereas pyrrole rings B and C are only slightly lifted up. The largest deviation in each ring is within 0.10−0.15 Å. The dihedral angle of the northern and southern least-squares planes is 5.1°. This value is close to that (2.9°) for the cobalamin in the substrate-bound enzyme·CN-Cbl complex (*10*), but far smaller than that (14.1°) for free CN-Cbl (*35*), indicating that the planarity of the corrin ring is not significantly affected by substrate binding.

Substrate Binding Site and Local Conformational Change upon Substrate Binding. In the substrate-bound enzyme, 1,2-propanediol is bound to the K^+ ion that is located in the $(\beta/\alpha)_8$ barrel of the α subunit (*9*) (Figure 3A). As expected, a 1,2-propanediol molecule is no longer bound to the K^+ ion in the substrate-free enzyme. Instead, two water molecules are found in the positions that are occupied by the hydroxyl groups of the 1,2-propanediol molecule in the substrate-bound form (Figure 3B,C). This indicates clearly that they coordinate to the K^+ ion in the same manner as the hydroxyl groups of 1,2-propanediol do in the substrate-bound form. These water molecules are named W1 and W2, corresponding to O1 and O2 in 1,2-propanediol, respectively. Both W1 and W2 are held by hydrogen bonding with two amino acid residues (Figure 3C): W1−OE2(Gluα170), W1−NE2(Glnα296), W2−NE2(Hisα143), and W2−OD2(Aspα335). Corresponding hydrogen bonds between the substrate hydroxyls and these amino acid residues have been found in all the previously determined substrate-bound structures (*9*, *10*).

Another water molecule, W3, is bound to both W1 and W2 through two hydrogen bonds (Figure 3B,C). W3 is held only by the hydrogen bonds, because there is no hydrogen bonding with adjacent residues or water molecules except W1 and W2. W3 might be there in the enzyme·AdoCbl complex, because the adenosyl group does not contact it as judged from a modeling study. Propionaldehyde that is formed may be displaced from the K^+ ion by a water molecule. Although it is not clear that W3 exists in an aldehyde-bound form of diol dehydratase, W3 or the water molecule bound near the W3 site might play this role in the release of the aldehyde product from the active site.

The methyl group of 1,2-propanediol pushes both the phenyl group of Phe374 and the amide group of Gln336 (Figure 4A,B). These interactions cause a slight rotation of the phenyl group (Figure 4A) and push down the Phe374/Ala375 moiety, which in turn pushes the *g*-acetamide side chain on pyrrole ring D (Figure 4C). This would lead to the tilt of the corrin ring of cobalamin, and as a result, the β subunit is also tilted by ∼3°. Thus, it seems clear that the substrate-induced local conformational change around the substrate-binding site causes domain movement upon substrate binding, which is responsible for the further labilization of the Co−C bond of the holoenzyme. The relatively low activity of 1,2-ethanediol may be reasonably explained by the absence of its methyl group.

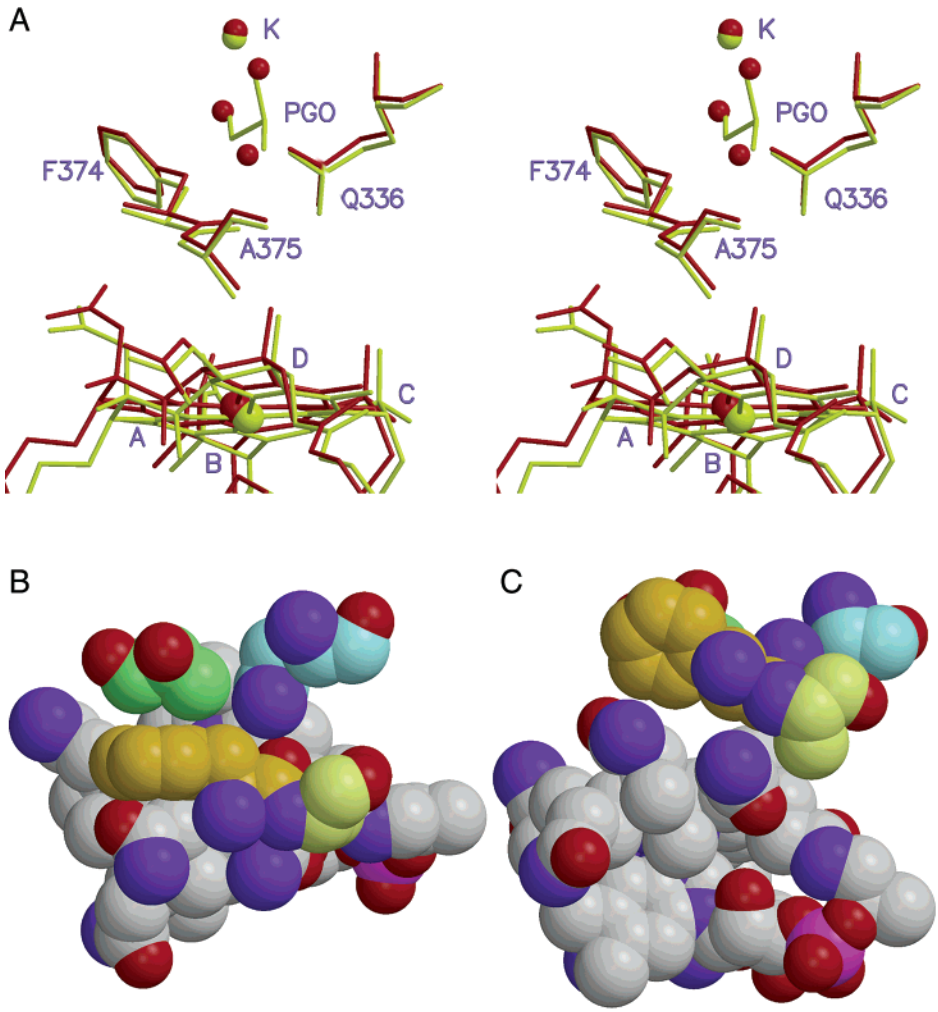

FIGURE 4: Local conformational change upon substrate binding. (A) Stick models showing conformational changes of cobalamin and the residues in contact with C(3) of 1,2-propanediol. Color codes are the same as in Figure 2B. Labels close to the corrin ring represent the names of pyrrole rings. (B) CPK models showing contacts between 1,2-propanediol and Pheα374 and between 1,2-propanediol and Glnα336. Color codes for carbon atoms were as follows: gray for cobalamin, green for 1,2-propanediol, cyan for Glnα336, orange for Pheα374, and yellow for Alaα375. Oxygen and nitrogen atoms of all the residues, cobalamin, and 1,2-propanediol are shown in red and blue, respectively. (C) CPK models showing contacts between Pheα374 and cobalamin and between Alaα375 and cobalamin. Color codes are the same as for panel B.

Channel for Substrate Uptake. In a previous paper (*9*), we considered a neutral hydrophilic residue-rich area (Asnα369, Asnα372, Asnβ188, Glnα336, Glnβ156, and Glnβ190) as a possible path for substrate uptake and product release. However, this possibility was eliminated here, because it turned out that the residues in this region do not move significantly upon substrate binding. Instead, we found a solvent channel through which the substrate-binding site can be directly accessible from outside of the enzyme (Figure 5A). The depth of the channel is approximately 15 Å. The channel runs through the C-terminal ends of strands 2 and 3 of the β barrel, a loop between residues Ileβ132 and Gluβ152, and the cobalamin. Side chains of four neutral hydrophilic residues (Thrα172, Serα202, Thrα207, and Serβ149) are found on the surface of the channel. These hydrophilic residues might guide a substrate molecule to the active site and a product to outside of the enzyme.

Surprisingly, the channel of the substrate-free enzyme is narrower than that of the substrate-bound one (Figure 5B). The movement of the β subunit upon substrate binding expands the channel. This seems strange since there are so many examples in which substrates close a cleft, channel, or cavity. In the case of this enzyme, the holoenzyme readily undergoes inactivation by oxygen in the absence of substrate. This inactivation is believed to be due to the reaction of the highly activated Co−C bond of the coenzyme with oxygen (*36*). Therefore, one of the most plausible explanations for this unusual feature might be that the closed conformation slows the intrusion of oxygen and thus the resulting oxygen inactivation of the holoenzyme.

K^+ Ion Binding Sites. In addition to the K^+ ion (K1) in the substrate binding site, two noncatalytic K^+ ions are found in the substrate-free enzyme (Figure 6A). One of them (K2) is located close to the adenine binding site as found in the enzyme·adeninylpentylcobalamin complex (*10*). It is hexacoordinated by O(Glyα261), OG(Serα264), OE1(Gluα265), OE1(Gluα280), and two water molecules (Figure 6B). The other one (K3) is located close to the water channel (Figure 6C). This K^+ ion is pentacoordinated with O(Leuα203), OE1(Gluα205), OE1(Gluα208), O(Thrα222), and OG1(Thrα222). An exchangeable water molecule might coordinate to it as a sixth ligand. In the substrate-bound form of the enzyme·adeninylpentylcobalamin complex, we assigned a water molecule for the electron density at this site,

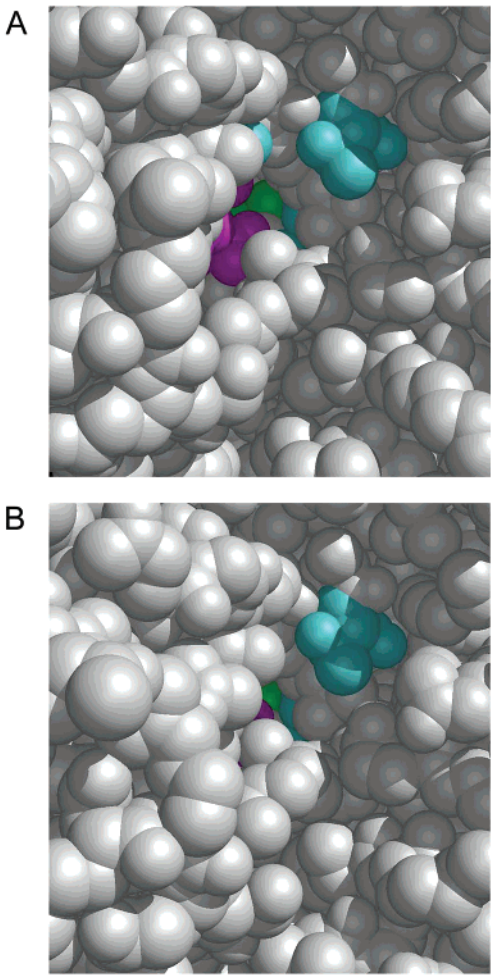

FIGURE 5: Solvent channel accessing the substrate binding site. Space-filling models created with MIDASPLUS (*45*, *46*): (A) substrate-bound form and (B) substrate-free form. Four neutral hydrophilic residues (Thrα172, Serα202, Thrα207, and Ser*β*149) are colored cyan. Magenta spheres are CN-Cbl. The substrate (yellow-green) in panel B is based on that in the substrate-bound form.

because the electron density was as high as that of oxygen (*10*). However, the finding of the third K^+ ion (K3) implies that it might not be a water molecule but an ammonium ion. That is, an ammonium ion derived from the reservoir solution might have replaced the K^+ ion, indicating that this site is easily accessible to monovalent cations through the solvent channel.

Modeling Study of the Substrate-Induced Labilization of the Co−C Bond. In a previous study (*10*), we identified the binding site for the adenine ring of the coenzyme adenosyl group in the structure of the substrate-bound form of the diol dehydratase·adeninylpentylcobalamin complex. The adenine moiety of the adeninylpentylcobalamin is held tightly in its binding pocket by hydrogen bonding at N(1) with H_2O, at N(3) with the side chain OH group of Serα224, at 6-NH_2 with main chain amide oxygens of Glyα261 and Serα299, and at N(7) with the main chain NH group of Serα301 (*10*). The structure of the adenine ring binding pocket provides a molecular basis for the strict specificity of this enzyme for the coenzyme adenosyl group. Since the relative positions of the amino acid residues that constitute the adenine ring binding site essentially do not change upon substrate binding, a modeling study similar to that reported previously for the substrate-bound enzyme·adeninylpentylcobalamin complex (*10*) is possible. Angular strains and tensile force leading to Co−C bond cleavage could be evaluated by superimposing the structures of both the cobalamin moiety and the adenine ring of AdoCbl onto those of the enzyme-bound CN-Cbl and the adenine ring binding pocket with the Co−C bond cleaved and the Co−C distance kept to a minimum. The putative adenine ring-bound position in the enzyme·CN-Cbl complex was determined by superimposition of the present structure with that of the ternary complex of the enzyme, adeninylpentylcobalamin, and 1,2-propanediol (*10*), followed by energy minimization as described in Experimental Procedures. In the presence of substrate (Figure 7B,E), the Co−C distance of the coenzyme has to be increased to 3.11 Å and the Co−C bond has to lean toward pyrrole ring B. This leads to distortions of the C(5′)−Co−N22(ring B) and Co−C(5′)−C(4′) bond angles of 46.9° and 155.9°, respectively. These values coincided reasonably well with those evaluated previously from the modeling study using the X-ray structure of the substrate-bound enzyme·adeninylpentylcobalamin complex (*10*). This indicates that such a modeling study with the structure of the enzyme·CN-Cbl complex is also valid.

When the same modeling study was carried out using the structure of the substrate-free form of the diol dehydratase·CN-Cbl complex (Figure 7C,F), the Co−C distance of AdoCbl has to be increased to 2.98 Å and the Co−C bond has to lean toward pyrrole ring B. As a result, the C(5′)−Co−N22(ring B) and Co−C(5′)−C(4′) bond angles would be distorted to 59.4° and 159.6°, respectively. These values are far from those for free AdoCbl (1.97 Å, 84.2°, and 124.4°, respectively) (*37*) (Figure 7A,D) and rather close to those obtained with the substrate-bound form of the enzyme·CN-Cbl complex. Therefore, it was strongly suggested that the Co−C bond becomes largely activated (labilized) upon coenzyme binding to the apoenzyme even in the absence of substrate and undergoes homolysis through the substrate-induced conformational change of the enzyme.

Mechanistic Implications. On the basis of the structure of the substrate-free form of diol dehydratase, we propose here some revision of our overall mechanism for diol dehydratase (*10*). The structure of the substrate-free enzyme revealed that two oxygen atoms of two water molecules coordinate to the K^+ ion instead of the substrate hydroxyls. That is, the K^+ ion in the substrate-free enzyme is also heptacoordinated by five oxygen atoms from the active site amino acid residues and two oxygen atoms of water molecules. As judged from the modeling study using the structure of the substrate-free enzyme, it is likely that major conformational changes occur upon coenzyme binding even in the absence of substrate that lead to activation of the Co−C bond of the coenzyme. At this stage, however, only a small fraction of the enzyme-bound coenzyme is in the dissociated form. In the absence of substrate, the adenosyl radical in the "proximal" conformation (Figure 1C) (*10*, *38*) would recombine rapidly with cob(II)alamin even if Co−C bond homolysis takes place, and thus, the fraction of the enzyme-bound coenzyme in the dissociated form would be kept very small. The addition of substrate to the holoenzyme brings about a ligand exchange reaction with two water molecules on the K^+ ion. When the substrate binds, rather small but distinct conformational changes take place that trigger Co−C bond homolysis

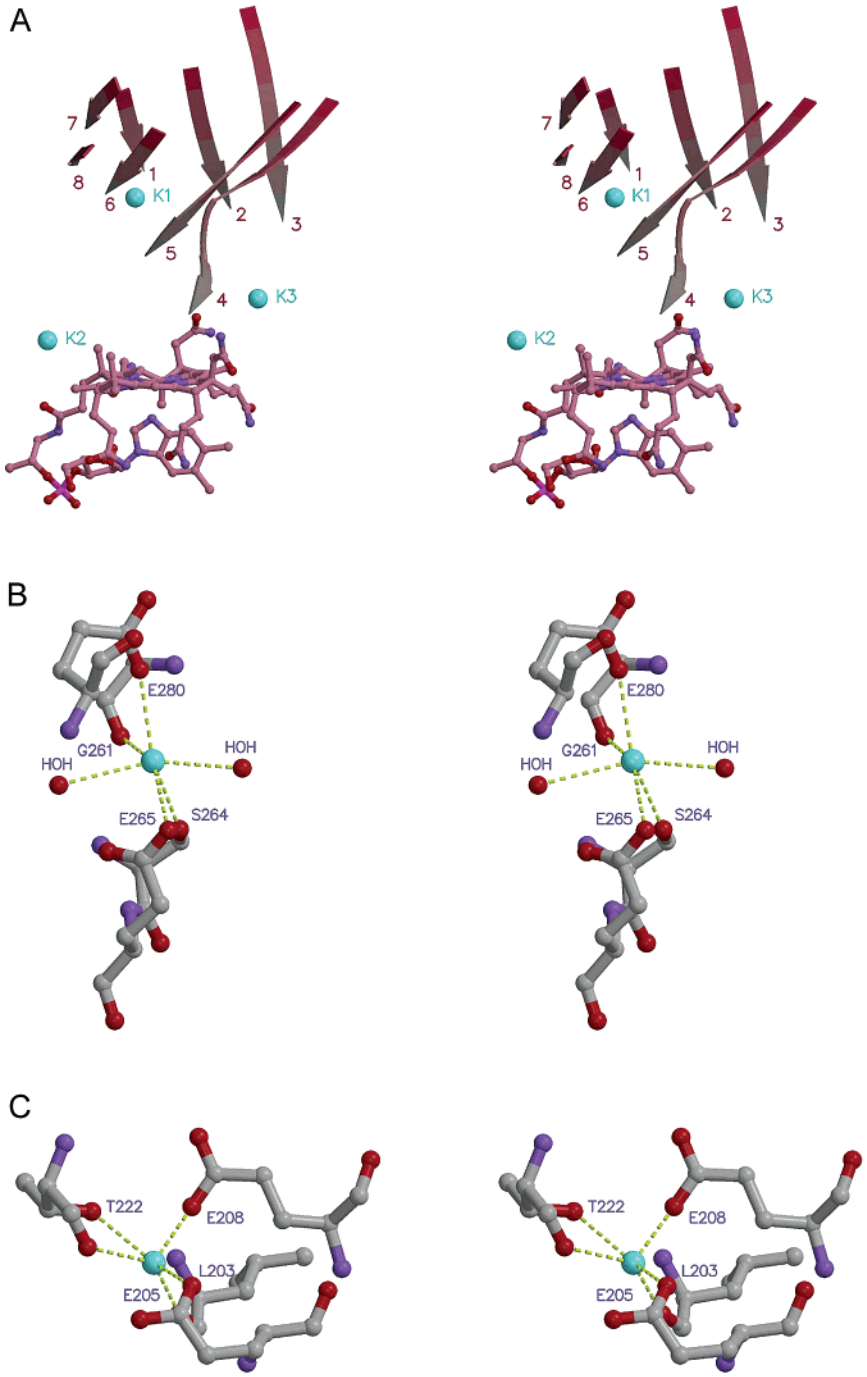

FIGURE 6: K^+ ion binding sites. (A) Overall view showing the relative positions of the three K^+ ions. K1−K3 show K^+ ions at the substrate binding site, at the adenine binding site, and near the water channel, respectively. The adjacent CN-Cbl and the β barrel of the α subunit are also depicted. (B) Close-up view of the second site (K2) adjacent to the adenine binding site. (C) Close-up view of the third site (K3). The color codes in panels B and C are the same as those in Figure 3C.

forming cob(II)alamin and the adenosyl radical. The adenosyl radical in the proximal conformation experiences a 94° counterclockwise rotation of the ribosyl moiety around the glycosidic linkage to the "distal" conformation (Figure 1D) (*10*, *38*). The C(5′)-centered radical of the ribosyl moiety now comes close to the specific hydrogen atom on C(1) of the substrate and abstracts it stereospecifically, forming 5′-deoxyadenosine and a substrate-derived radical. According to our density functional theory computation with a protein-free, simplified model of the diol dehydratase system, the relative energy is lowered upon this hydrogen abstraction by 6.7 kcal/mol with an activation energy of 9.0 kcal/mol (*38*, *39*). Thus, the equilibrium would shift to the direction of Co−C bond homolysis. Kinetic coupling of Co−C bond cleavage and hydrogen abstraction was first shown by the substrate deuterium KIE on the cleavage rate with methylmalonyl-CoA mutase (*40*), glutamate mutase (*41*), and ethanolamine ammonia lyase (*42*). We also observed the substrate deuterium KIE on the rate of Co−C bond homolysis with diol dehydratase (M. Yamanishi and T. Toraya, manuscript to be published). Therefore, it is evident in diol dehydratase as well that the equilibrium is shifted in favor of Co−C bond homolysis by its kinetic coupling with the hydrogen abstraction from the substrate.

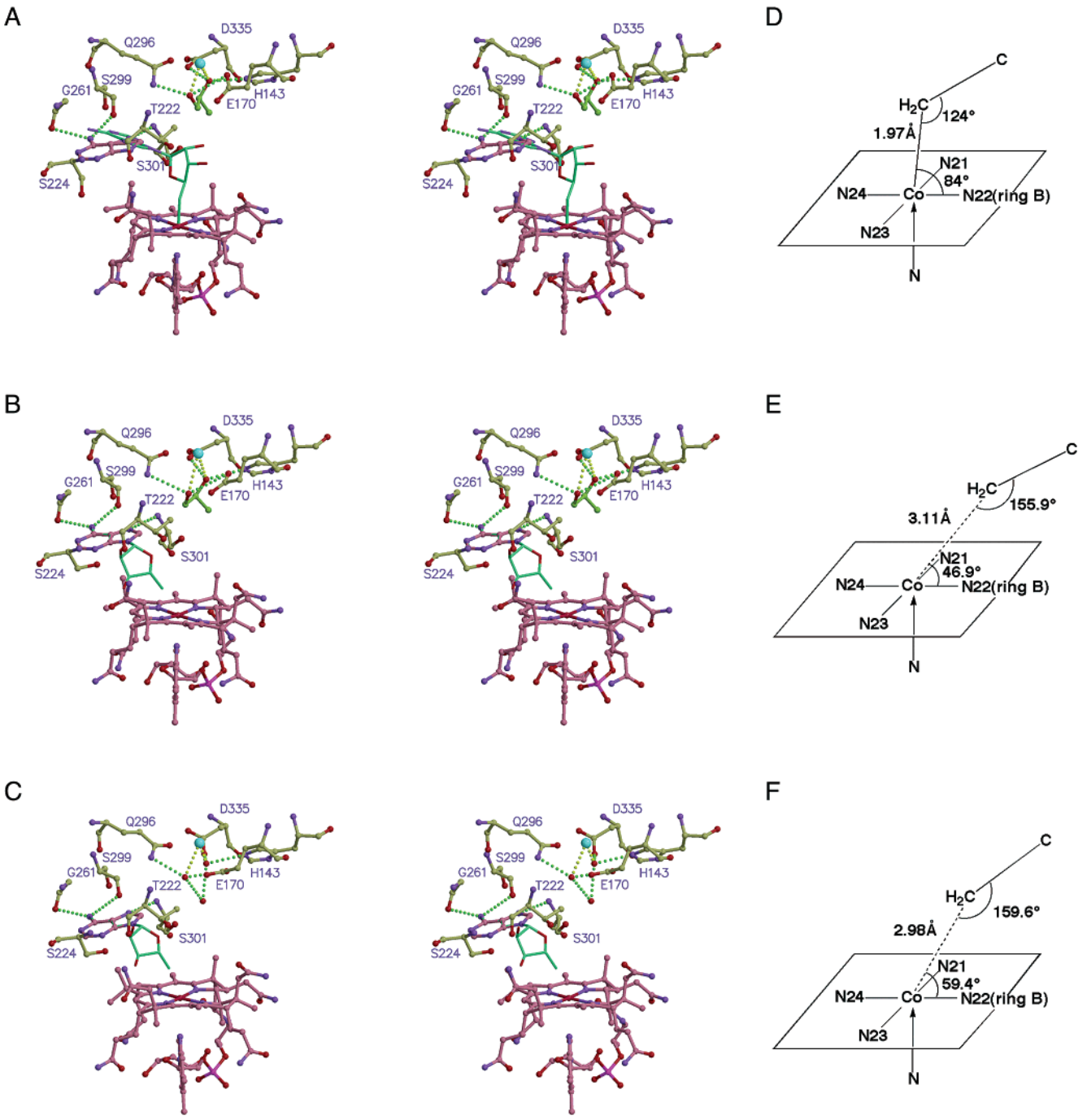

FIGURE 7: Modeling study of the superimposition of the structure of AdoCbl on those of the substrate-bound and substrate-free forms of the enzyme·CN-Cbl complex. The adenine ring is derived from the structure of the adeninylpentylcobalamin complex (*10*). The adenosyl group of the coenzyme is shown as a green stick model. (A) Substrate-bound form with the AdoCbl superimposed at the cobalamin moiety without cleavage of the Co−C bond. (B) Same as panel A, but with the Co−C bond cleaved and the Co−C distance kept to a minimum. (C) Substrate-free form. The adenosyl group was treated in the same way as described for panel B. The methods of energy minimization for panels B and C are described in the text.

In contrast to the rather small conformational change of diol dehydratase when the substrate binds, substrate binding induces a marked conformational change in the case of methylmalonyl-CoA mutase (*11*); that is, the so-called TIM (triose phosphate isomerase) barrel is split apart. Such a large change needs a larger substrate binding energy and thus might be able to be induced only by the binding of a large substrate, such as CoA derivatives. The inactivation of holoenzymes of diol dehydratase (*36*) and glycerol dehydratase (*43*) by oxygen in the absence of substrate has been postulated to be due to the irreversible cleavage of the activated Co−C bond of the holoenzyme by reaction with oxygen (*36*, *44*). In contrast, resonance Raman spectra showed that the Co−C bond is left unaffected when the coenzyme binds to methylmalonyl-CoA mutase in the absence of substrate (*16*). Such different effects of substrate

binding on Co−C bond cleavage might be reminiscent of the difference in the substrate-induced conformational change between these enzymes.

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