Radical production simulated by photo-irradiation of the diol dehydratase–adeninylpentylcobalamin complex

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In the course of structural studies of diol dehydratase–cobalamin complexes, it was found that the electron density corresponding to the cyano group of the enzyme-bound cyanocobalamin is almost not observable at room temperature and very low even at cryogenic temperatures, suggesting its dissociation from the Co atom upon X-ray irradiation. On the contrary, the adenine moiety of the enzyme-bound adeninylpentylcobalamin was clearly located in the electron density map. When the enzyme–adeninylpentylcobalamin complex was illuminated with visible light, the electron density between the C5′ and Co atoms disappeared, and the temperature factors of the atoms comprising the pentamethylene group became much larger than those in the dark. This indicates a Co—C bond cleavage and that the adenine moiety remains held by hydrogen bonds with some residues in the enzyme. Thus, the formation of an adenine-anchored radical upon illumination was demonstrated crystallographically with this complex. These observations clearly indicate that homolysis of the Co—C bond of alkylcobalamin takes place upon illumination with visible light but is not readily cleaved during X-ray irradiation.

Keywords: coenzyme B_{12}; adenosylcobalamin; B_{12} enzyme; diol dehydratase; adenine-anchored radicals.

1. Introduction

Adenosylcobalamin (coenzyme B_{12}) (Fig. 1*a*) is a naturally occurring organometallic compound of complex structure and serves as a cofactor for enzymatic radical reactions (Toraya, 2000*a*). Diol dehydratase is one of the representative B_{12} enzymes that catalyzes the conversion of 1,2-diols into the corresponding aldehydes (Fig. 1*c*) (Toraya, 2000*b*). The reaction is initiated by homolytic cleavage of the Co—C bond of the coenzyme and proceeds *via* a radical mechanism. The minimal mechanism as well as the structures of the coenzyme and its analogues are illustrated in Fig. 1. The enzyme is a $\alpha_2\beta_2\gamma_2$ heterooligomer and absolutely requires a potassium ion for catalytic activity.

We have previously determined the crystal structure of the diol dehydratase–cyanocobalamin complex at 277 K (Shibata *et al.*, 1999). This enzyme binds cobalamin in the so-called 'base-on' mode, *i.e.* with the dimethylbenzimidazole moiety coordinating to the Co atom (Fig. 1*e*). Some of the other B_{12} enzymes bind it in the 'base-off' mode with histidine ligation (Drennan *et al.*, 1994; Mancia *et al.*, 1996; Reitzer *et al.*, 1999) (Fig. 1*d*). The electron density of the CN group, the upper axial ligand of cyanocobalamin (Fig. 1*f*), was not clear in the structure. In the structure of methionine synthase, which was also determined at 277 K, the election density of the methyl group could not be observed in the figure reported (Drennan *et al.*, 1994). On the contrary, in the case of glutamate mutase, which was studied at cryogenic temperature, the electron densities of the methyl and CN groups were partly observable in the complexes with methylcobalamin and cyanocobalamin, respectively (Reitzer *et al.*, 1999). They concluded that their structure contained ~50% cobalamin in the cyano- or methyl-Co(III) state and 50% in a reduced state. From this point of view, and in order to understand the mechanism of enzyme action, crystal structure analyses of diol dehydratase–cyanocobalamin and diol dehydratase–adeninylpentylcobalamin complexes at cryogenic temperature were carried out and reported (Masuda *et al.*, 2000) (Fig. 2).

Adeninylpentylcobalamin is an inactive analogue of adenosylcobalamin having an adenine ring in the upper axial ligand (Fig. 1*g*) and bound more tightly than adenosylcobalamin by diol dehydratase (Toraya *et al.*, 1977) and some of the other cobalamin-dependent enzymes (Sando *et al.*, 1976; Krouwer *et al.*, 1980). This analogue is believed to bind to the active sites of enzymes in a similar manner as the regular coenzyme, but to require less energy for accommodation owing to flexibility of the pentamethylene group. The X-ray structures enabled us to identify the adenine-binding site of the enzyme and revealed the exact details of enzyme–cobalamin interactions as well as conformations of enzyme and cobalamin. Modelling studies were also conducted to elucidate the mechanism of the Co—C bond cleavage of the coenzyme and to explain the reported stereospecificity of the hydrogen abstraction. Based on these, a detailed discussion concerning the mechanism of action of this enzyme has become possible.

In the course of our studies, we recognized that the upper axial ligand of cobalamin behaved differently in each complex. The electron density of the cyano group was smeared out, suggesting this group mostly dissociated from the Co atom. On the contrary, the adeninylpentyl group remained bound to the Co atom under the dark condition, suggesting synchrotron radiation has little effect on the Co—C bond of the enzyme-bound adeninylpentylcobalamin. These observations led us to carry out the X-ray experiments under conditions illuminated with visible light. Here we wish to report the results of these experiments.

2. Experimental

Diol dehydratase from *Klebsiella oxytoca* was expressed in *Escherichia coli* and purified to homogeneity as described previously (Tobimatsu *et al.*, 1997). Crystals of the complex composed of apodiol dehydratase, cyanocobalamin and 1,2-propanediol were grown by the sandwich-drop vapour diffusion method at 277 K, as described previously (Masuda *et al.*, 1999). Crystals of the diol dehydratase–adeninylpentylcobalamin complex were also grown under the same conditions in the presence of 3.0 μ*M* adeninylpentylcobalamin instead of cyanocobalamin. For cryogenic data collection, all crystals were transferred into a solution containing 18% polyethylene glycol (PEG) 20000, 0.24 *M* ammonium sulfate, 0.30% lauryl dimethylamine oxide (LDAO), 2.0% 1,2-propanediol, 10 m*M* potassium phosphate buffer (pH 8.0), 20 m*M* tris-HCl buffer (pH 8.0) and 20 μ*M* cyanocobalamin or 3.0 μ*M* adeninylpentylcobalamin. The solution containing 25% (*v*/*v*) ethylene glycol was gradually added to the drop containing crystals to a final ethylene glycol concentration of 17.5% (*v*/*v*). PEG with a higher molecular weight than that in the crystallization condition was used to compensate the increase of protein solubility upon addition of ethylene glycol. X-ray experiments were carried out on beamline BL44B2 at SPring-8, Japan. Diffraction data were recorded with a 185 mm-diameter Mar CCD with X-rays of

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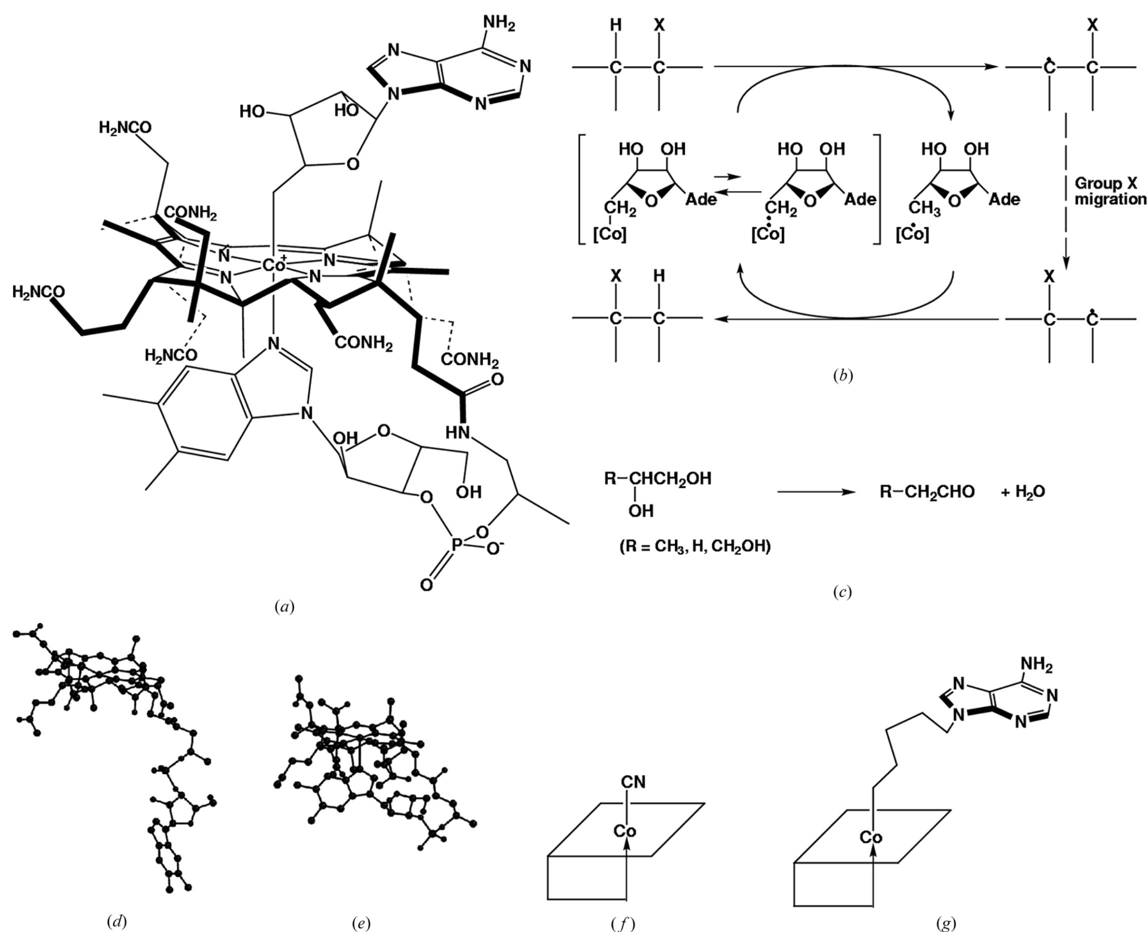

Figure 1

Adenosylcobalamin and its analogs, modes of cobalamin binding, and the reaction mechanism catalyzed by adenosylcobalamin-dependent enzymes. (*a*) Three-dimensional structure of adenosylcobalamin (coenzyme B_{12}). (*b*) The minimal mechanism for adenosylcobalamin-dependent rearrangements: [Co], cobalamin; Ade, 9-adeninyl; X, a generic migrating group. (*c*) The reaction catalyzed by diol dehydratase. The hydroxyl group migrates from C(2) to C(1). (*d*) Base-off mode of cobalamin binding (methylmalonyl-CoA mutase). (*e*) Base-on mode of cobalamin binding (diol dehydratase). (*f*) Cyanocobalamin. (*g*) Adeninylpentylcobalamin. The parallelograms in (*f*) and (*g*) represents a corrin ring of cobalamins.

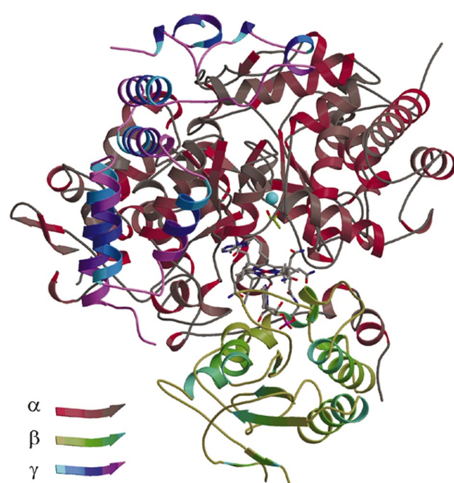

Figure 2

A schematic drawing of the diol dehydratase–adeninylpentylcobalamin complex showing $\alpha\beta\gamma$ hetero-trimer. Colours ranging from crimson to grey are used for the α subunit, from khaki to cyan for the β subunit, and from cyan to violet for the γ subunit. Cyanocobalamin is located in the interface between α and β subunits. The blue ball indicates an essential potassium ion. The substrate, propanediol, is found to be coordinated to the potassium ion. The colour key for each subunit is shown.

0.7 Å and a total exposure time of 900 s for a set of complete data. Since adeninylpentylcobalamin is unstable to visible light, a series of experiments from the preparation of crystals to X-ray data measurement were carried out in a dark environment. A safety red light was used to pick up a crystal and to mount it on a goniometer head of the X-ray equipment. Data collection, with the diol dehydratase–adeninylpentylcobalamin complex under illuminated conditions, was performed as follows. The crystal used for data collection under dark conditions was illuminated for 15 min by a halogen lamp (150 W) of optical fibre light source manufactured by Nikon Co., Japan, placed at a distance of 100 mm from the crystal; then the X-ray experiment was carried out. During data collection, the crystal was continuously illuminated. The light was then turned off and the crystal was kept under dark conditions for 15 min. Then, a third data collection was carried out in a completely dark environment. This procedure was repeated. The whole experiment is illustrated in Fig. 3.

The structure of the illuminated diol dehydratase–adeninylpentylcobalamin complex was refined using the program *SHELXH* (Sheldrick & Schneider, 1997) using data of resolution between 30 and 1.75 Å. The starting model was the partially refined structure determined at 1.70 Å resolution in the dark (100 K). The restraints for bond lengths and bond angles in the pentamethylene moiety were applied throughout the refinement. When the restraints

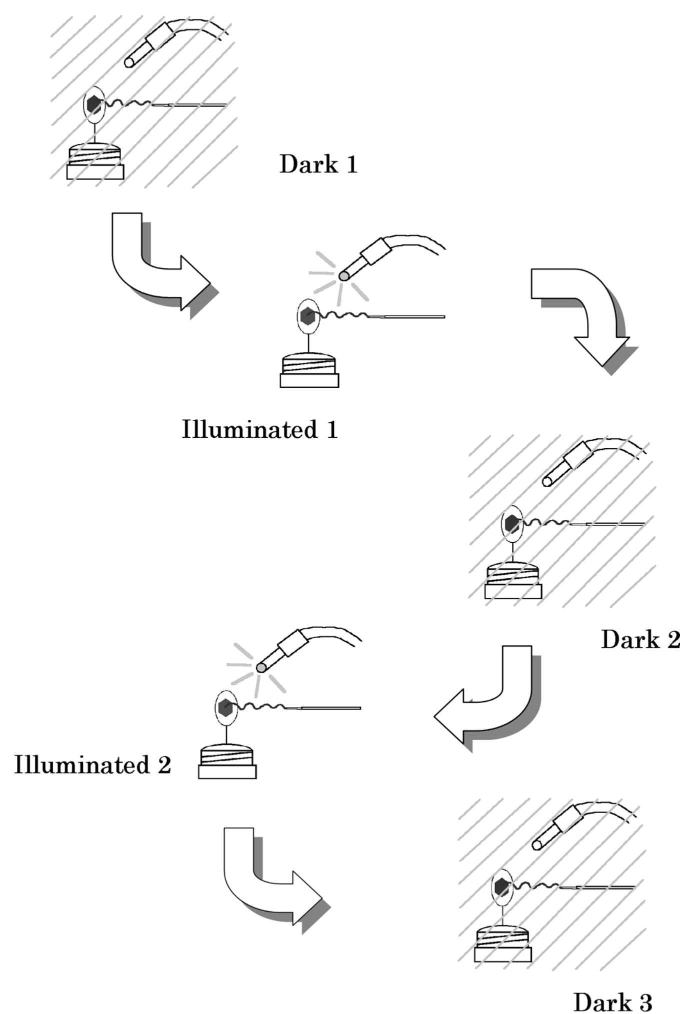

Figure 3
A flow diagram of the whole experiment. The first diffraction data set was collected under dark conditions. The second diffraction experiment was carried out under illuminated conditions. The following experiments were carried out as shown.

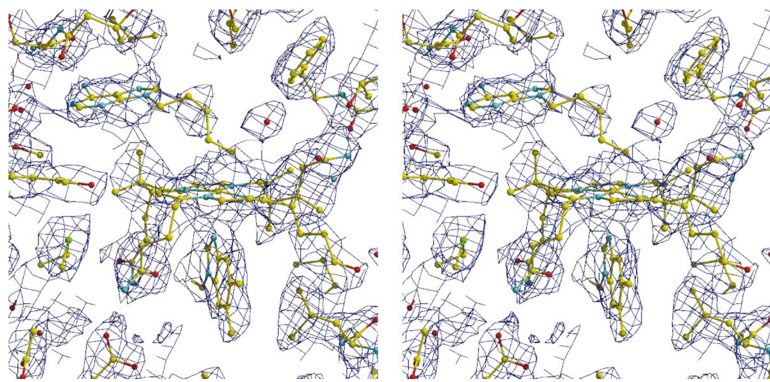

Figure 4
A stereo pair of part of the electron density map contoured for residues around the adeninylpentylcobalamin in the diol dehydratase–adeninylpentylcobalamin complex under the secondly illuminated condition (1.75 Å resolution). The blue contours are drawn for the $(2mF_o - DF_c)$ map. A stick model of the unphotolyzed molecule is superimposed. Note that the electron density corresponding to the pentyl moiety has almost disappeared.

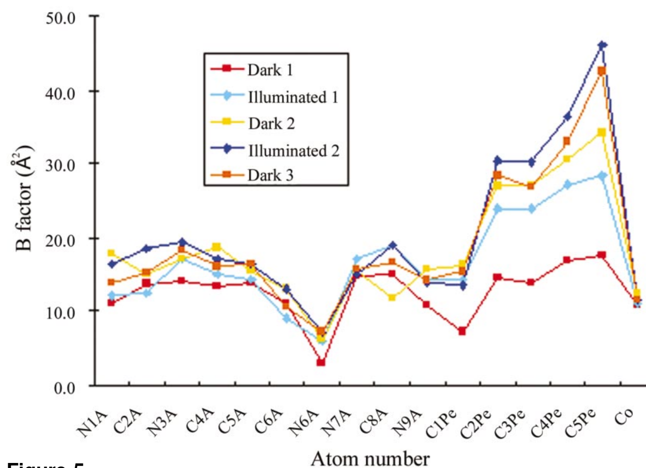

Figure 5
Position-dependent changes in B-factors in the adeninylpentyl group before and after photoirradiation.

were not applied to the dark structure, the structure of the pentamethylene moiety was thoroughly distorted. In the case of the illuminated structure, refinement without restraints resulted in missing all C atoms in the pentamethylene moiety. As for the bond lengths between the Co atom and lower or upper axial ligand, the refinement was carried out without restraint for bond distances and bond angles. The data sets followed were processed in a similar way.

3. Results and discussion

It has been established that illumination with visible light causes homolysis of the Co—C bond of alkylcobalamins (Hogenkamp, 1982). In the case of adeninylpentylcobalamin, an adeninylpentyl radical is formed. The electron density maps around the cobalamin moiety of the enzyme–adeninylpentylcobalamin complex for the secondly illuminated stage is shown in Fig. 4. The electron density between the C5′ and Co atoms disappeared upon illumination. Temperature factors of the atoms comprising the adeninylpentyl group are plotted for all stages in Fig. 5. These figures clearly indicate that the bond between the pentamethylene group and the Co atom is cleaved by photoirradiation, whereas the adenine moiety remains held by hydrogen bonds with some residues in the α subunit. Thus, the formation of an adenine-anchored radical upon photoirradiation was highly suggested crystallographically for the enzyme–adeninylpentylcobalamin complex. Although the formation of an adenine-anchored radical under photolytic conditions mimics the initial stage of the diol dehydratase catalysis, no enzymatic reaction followed it. Previous X-ray structure analysis of the enzyme revealed that the substrate binds far away from the Co atom (Shibata *et al.*, 1999). Thus, this would be partly because the radical centre of the adeninylpentyl radical could not come close to the substrate owing to the lack of the β-D-ribofuranose ring and its functional groups.

The electron density map around cobalamin of the enzyme–cyanocobalamin complex at 100 K has already been shown (Masuda *et al.*, 2000). It is evident that the electron density of the cyano group is partly visible at cryogenic temperatures, whereas it was almost invisible in the 277 K structure. The bond distances between Co and N(3) of 5,6-dimethylbenzimidazole in the 277 K and 100 K structures are 2.50 and 2.18 Å, respectively.

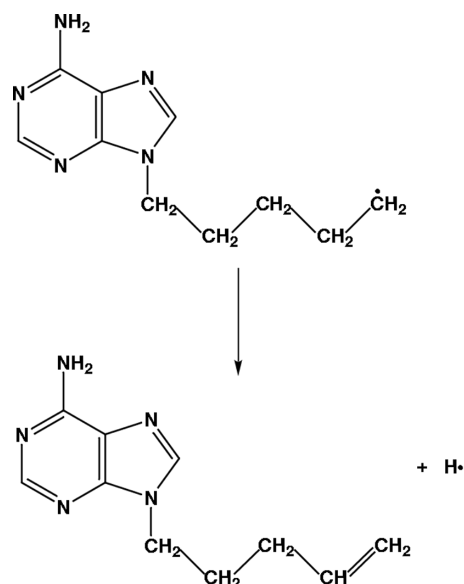

Figure 6
Possible stabilization of the adenosyl radical by dehydrogenation.

For the adeninylpentylcobalamin complex, the situation is very different. In the electron density map, the adenine moiety is clearly visible even after the repeated illumination, whereas the pentamethylene group is visible before illumination but smeared out after illumination. These observations led us to the following idea. The Co—C bond of the enzyme-bound adeninylpentylcobalamin is readily cleaved by visible light to give the corresponding radicals. However, the adenine moiety of the adeninylpentyl radical is tightly held near the original position by the hydrogen-bond network between the four N atoms and nearby groups in the protein. Therefore the adeninylpentyl radical and divalent cobalt ion could recombine to regenerate adeninylpentylcobalamin. It should be noted in Fig. 5 that the photolytic dissociation of the Co—C bond is not fully reversible. Repeated illumination resulted in the increase of the fraction of the irreversibly cleaved Co—C bond, probably because the pentamethylene group is very flexible and some fractions of the radical might take the conformation that is not suitable for the recombination. It was reported that photolysis of alkylcobalamins under anaerobic conditions results in the formation of alkenes with reduction of cob(II)alamin to cob(I)alamin (Dolphin *et al.*, 1964). This suggests that the adeninylpentyl radical may undergo stabilization by dehydrogenation to 5-adeninyl-1-pentene, as illustrated in Fig. 6. Since this alkene is no longer reactive towards reduced cobalamins under neutral conditions, continuous photoirradiation would induce the irreversible cleavage of the Co—C bond.

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