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Essential Roles of Nucleotide-Switch and Metal-Coordinating Residues for Chaperone Function of Diol Dehydratase-Reactivase

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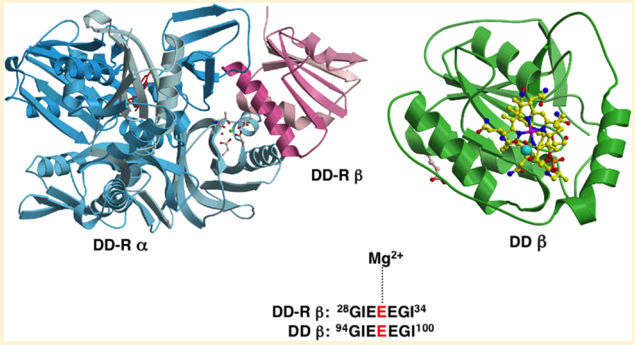
ABSTRACT: Diol dehydratase-reactivase (DD-R) is a molecular chaperone that reactivates inactivated holodiol dehydratase (DD) by cofactor exchange. Its ADP-bound and ATP-bound forms are high-affinity and low-affinity forms for DD, respectively. Among DD-Rs mutated at the nucleotide-binding site, neither the Dα8N nor Dα413N mutant was effective as a reactivase. Although Dα413N showed ATPase activity, it did not mediate cyanocobalamin (CN-Cbl) release from the DD·CN-Cbl complex in the presence of ATP or ADP and formed a tight complex with apoDD even in the presence of ATP, suggesting the involvement of Aspα413 in the nucleotide switch. In contrast, Dα8N showed very low ATPase activity and did not mediate CN-Cbl release from the complex in the presence of ATP, but it did cause about 50% release in the presence of ADP. The complex formation of this mutant with DD was partially reversed by ATP, suggesting that Aspα8 is involved in the ATPase activity but only partially in the nucleotide switch. Among DD-Rs mutated at the Mg^{2+}-binding site, only Eβ31Q was about 30% as active as wild-type DD-R and formed a tight complex with apoDD, indicating that the DD-R β subunit is not absolutely required for reactivation. If subunit swapping occurs between the DD-R β and DD β subunits, Gluβ97 of DD would coordinate to Mg^{2+}. The complex of Eβ97Q DD with CN-Cbl was not activated by wild-type DD-R. No complex was formed between this mutant and wild-type DD-R, indicating that the coordination of Gluβ97 to Mg^{2+} is essential for subunit swapping and therefore for (re)activation.



Enzymatic radical catalysis is defined as a catalytic mechanism by which enzymes catalyze chemically challenging reactions by utilizing highly reactive radicals.[1] Coenzyme B_{12} or adenosylcobalamin (AdoCbl)-dependent enzymes, such as diol dehydratase (DD) (EC 4.2.1.28), glycerol dehydratase (GD) (EC 4.2.1.30), and ethanolamine ammonia-lyase (EAL) (EC 4.3.1.7), are typical radical enzymes. They use an adenosyl radical, a primary carbon radical, which is formed by homolytic cleavage of the coenzyme cobalt−carbon (Co−C) bond.[1−4] Although enzymes generally deal with highly reactive intermediates by "negative catalysis",[5] these enzymes tend to undergo inactivation during catalysis with physiological substrates or even in the absence of substrate. The inactivation is due to undesired side reactions and accompanies the irreversible cleavage of the Co−C bond of the enzyme-bound coenzyme. The damaged cofactors thus formed remain tightly bound to apoenzymes and cannot be displaced by intact AdoCbl.

DD and GD are isofunctional enzymes that catalyze the conversion of 1,2-propanediol, glycerol, and 1,2-ethanediol to the corresponding aldehydes,[6−8] but they both undergo mechanism-based inactivation by glycerol.[7,9−11] In this inactivation, AdoCbl is converted to 5′-deoxyadenosine and an unidentified cobalamin[9] showing an alkylcobalamin-like spectrum that is gradually changed to a hydroxocobalamin (OH-Cbl)-like one by dialysis.[12] It was assumed from theoretical calculations[13] that glycerol inactivation begins with intramolecular hydrogen transfer from the glycerol 3-OH group to C1 of the substrate-derived radical to form an O3-centered radical that decomposes to formaldehyde and glycol radical, a possibility that should be experimentally tested. We reported previously that both glycerol-inactivated and O_2-inactivated holoenzymes of these dehydratases are rapidly reactivated in permeabilized bacterial cells (in situ)[14,15] and in vitro.[16−20] When the inactive complexes of DD with adenine-lacking cobalamins such as cyanocobalamin (CN-Cbl) and OH-Cbl are used as models of inactivated holoDD, they undergo activation under the same conditions.[14,15,17,18,20] Inactivated holoEAL is also reactivated in situ.[21] The specific protein factors responsible for their reactivation were first designated "reactivating factors"[16−22] and then renamed "reactivases" (DD-R, GD-R, EAL-R) because multiple turnovers were shown with DD-R.[23] DD-R and GD-R are molecular chaperones with low ATPase activities and enable reactivation of inactivated holoenzymes by cofactor exchange,[17,18,20,22−24] as shown in Figure 1A: reactivases bind ATP and hydrolyze it to ADP. The resultant ADP-bound forms

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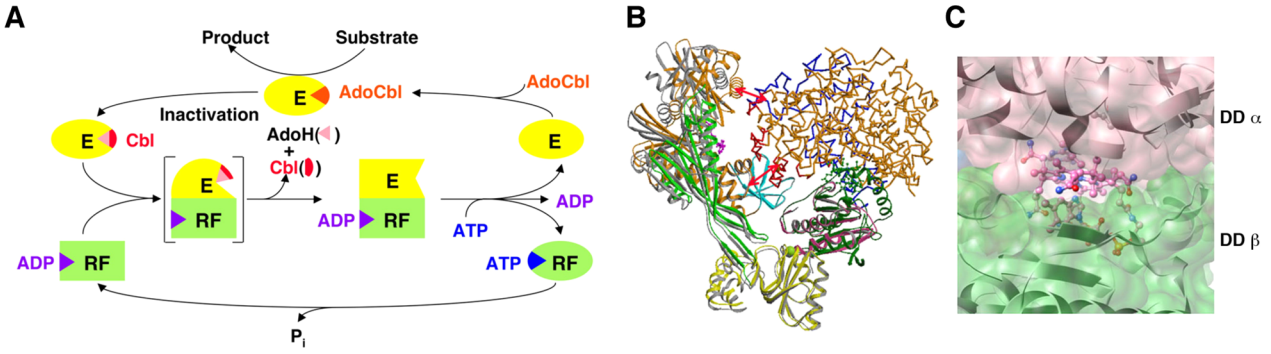

Figure 1. Reactivation of inactivated holoenzymes by reactivases. (A) Cofactor exchange mechanism. E, DD or GD; RF, DD-R or GD-R; X-Cbl, a damaged cofactor or an adenine-lacking cobalamin such as CN-Cbl or OH-Cbl; AdoH, 5′-deoxyadenosine. AdoH is released during reactivation. (B) Docking model of the DD·DD-R complex in which the DD β (green) and DD-R β (pink) subunits are superimposed. Red arrows show steric repulsion. (C) Cavity between the DD α (pink) and β (green) subunits. Panels B and C were drawn with Molscript[41]/Raster3D[42] and Chimera,[43] respectively.

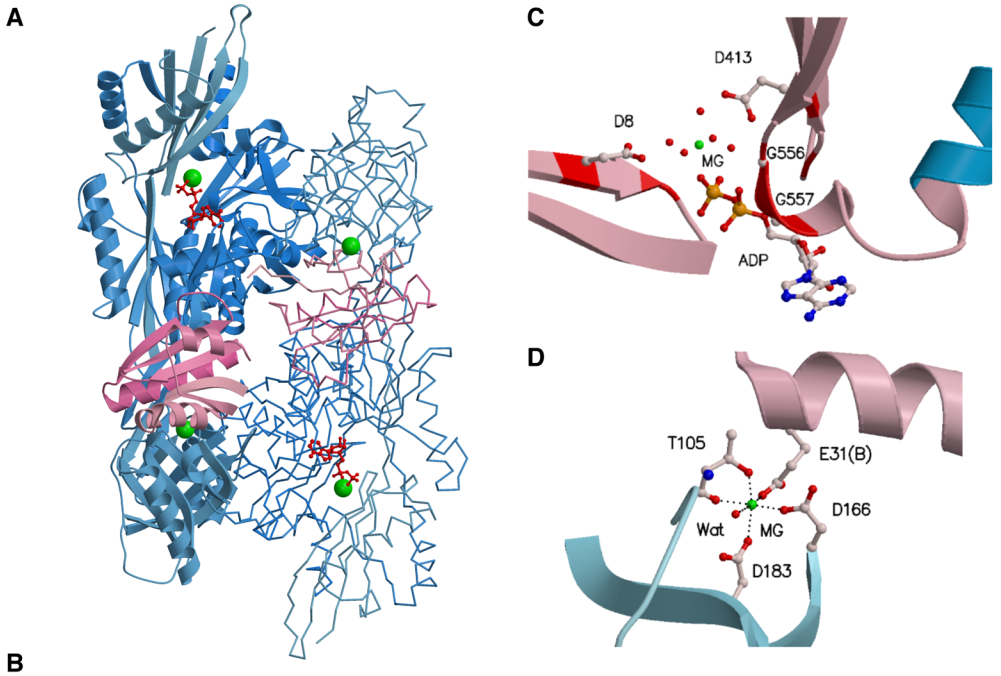

Figure 2. X-ray structure of DD-R in the ADP-bound form and the conserved regions among reactivases and Hsp70 family chaperones. (A) Overall structure $(\alpha\beta)_2$. The α and β subunits are colored blue and pink, respectively, darkening continuously from the N-terminal to the C-terminal sides. Green balls represent metal ions (Mg^{2+} in the ADP-bound form). (B) Fragmentary sequence homology between reactivases,[16,19,21] GlmL,[44] and Hsp70 family chaperones.[45,46] Asterisks and dots indicate identical and similar residues, respectively. Kox, *K. oxytoca*; Kpn, *Klebsiella pneumoniae*; Eco, *E. coli*; hum, human. (C) Nucleotide-binding site in the α subunit and mapping of the conserved regions on it. Red and pink indicate identical and similar residues, respectively. Red balls represent water molecules. (D) Metal ion-binding site in the interface between the α (blue) and β (pink) subunits. Residue numbers in the α subunit are shown. Panels A, C, and D were drawn with Molscript[41]/Raster3D.[42]

of reactivases have a high affinity for dehydratases and form tight complexes with apoenzymes in a nucleotide-dependent manner, which leads to concomitant release of damaged cofactors. Upon reversion of reactivases to a low-affinity form by replacement of bound ADP with free ATP, inactive apoenzyme·reactivase complexes dissociate into apoenzymes and reactivases. The formed apoenzymes are reconstitutable into active holoenzymes with intact AdoCbl. In contrast, active holoenzymes do not form complexes with reactivases, and thus, AdoCbl is not released. Adeninylpentylcobalamin, a cobalamin lacking adenosine but containing adenine, is also not released, indicating that reactivases discriminate a damaged cofactor from intact coenzyme by the presence of an adenine ring in the upper axial ligand.[17,18,20]

DD-R and GD-R are structurally similar and exist as a dimer of heterodimers $(\alpha\beta)_2$ [α, DdrA or GdrA (DhaF); β, DdrB or GdrB (DhaG)] (Figure 2A).[25,26] Their α subunits have a structural feature common to the ATPase domains of actin superfamily

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proteins including Hsp70 molecular chaperones, whereas the β subunits have folds similar to those of the β subunits of cognate dehydratases.[25,26] The binding of ADP to the nucleotide-binding site of DD-R causes a marked conformational change of the ATPase domain that would weaken the interactions between its α and β subunits and promote subunit swapping (i.e., the displacement of the DD-R β subunit by DD through the β subunit).[26] It was shown biochemically that such subunit swapping actually occurs upon DD·DD-R complex formation.[23] A modeled structure of the DD·DD-R complex (Figure 1B) suggests that induced steric repulsion between the DD-R α and DD α subunits results in tilting of the DD α subunit with respect to the DD β subunit, widening the preexisting cavity (~5 Å in height and ~15 Å in width; Figure 1C) to ~11 Å in height and allowing a damaged cofactor to be released from inactivated holoenzymes.

In this study, we introduced mutations into amino acid residues at two functional sites of DD-R, namely, the nucleotide-binding and metal ion-binding sites, and analyzed the impacts of these mutations on its chaperone function. In addition, the amino acid residue of DD that is essential for reactivation by DD-R is also reported here.

MATERIALS AND METHODS

Materials. Crystalline AdoCbl was a gift from Eisai Co. Ltd. (Tokyo, Japan). CN-Cbl was obtained from Glaxo Research Laboratories (Greenford, UK). [γ-^{32}P]ATP was obtained from PerkinElmer, Inc. (Waltham, MA). All other chemicals were commercial products of the highest grade available and were used without further purification. Recombinant DD and its reactivase, DD-R, of *Klebsiella oxytoca* were purified to homogeneity from overexpressing *Escherichia coli* JM109 strain harboring expression plasmid pUSI2E(DD)[27] and *E. coli* JM109 strain harboring expression plasmid pUSI2ENd(6/5b),[17,18] respectively, as reported previously. DD and DD-R mutants were purified to homogeneity from overexpressing *E. coli* JM109 strain harboring the mutant plasmid of pUSI2ENd-(DD)[28] and *E. coli* JM109 strain harboring the mutant plasmid of pCXV(5b-6),[16] respectively, by the same procedure as described for the wild-type proteins.

Construction of Mutant DD-Rs. Standard recombinant DNA techniques were performed as described by Sambrook et al.[29] Mutations were introduced into pCXV(5b-6) using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used to introduce mutations are listed in Table 1. It was confirmed by sequencing of the DNA region encompassing the entire *ddrA* and *ddrB* genes that no unintended mutations were incorporated during mutagenesis.

Table 1. Oligonucleotides Used for Site-Directed Mutagenesis[a]

target gene	mutation(s)	sequence (sense primers)
ddrA	D8N	cgatatatagctggcatt*aac*atcggcaactcatcgacg
ddrA	T105A	cataacccgaaag*cg*cccggcggcgcg
ddrA	D166A	cattttacagcgc*gc*cgatggcgtgctgg
ddrA	D183A	gctgccgatcgttg*cc*gaagtgctgtacatc
ddrA	D413N	ctggcgatcctc*aac*ctcggcgcgg
ddrA	G556A, G557A	gtggtgctggtc*gc*c*gc*ctcgtcgctggatttc
ddrB	E31Q	ctgctgggtatcgaa*ca*ggaaggtatccctttc
pddB	E96Q, E97Q, E98Q	gtcattgccggtatt*caacaacaa*ggcattaaggcgcgcg
pddB	E97Q	gtcattgccggtattgaa*caa*gaaggcattaaggc

[a]Italics indicate codons corresponding to the substituted amino acid residues. The oligonucleotides having the complementary sequences in the opposite direction were used as the respective antisense primers.

Construction of Mutant DDs. Mutations of the expression plasmids for DD were also introduced using a QuikChange site-directed mutagenesis kit. pUSI2ENd(DD) was used as a template. The primers used for mutagenesis are listed in Table 1. To confirm that no unintended mutations were incorporated during mutagenesis, the DNA region encompassing the entire *pddA*, *pddB*, and *pddC* genes was sequenced.

In Situ Reactivation and Activation Assays. *E. coli* JM109 was cotransformed with pUSI2E(DD) and pCXV(5b-6) containing mutated *ddrA* or *ddrB* genes or pUSI2ENd(DD) containing mutated *pddB* gene and pCXV(5b-6). Co-transformed cells were induced by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C. The cell suspension at a concentration of 10 mg of dry cells/mL in 0.05 M potassium phosphate buffer (pH 8.0) (buffer A) containing 2% 1,2-propanediol was treated with 1% toluene and then washed with buffer A as described previously.[14,16]

Toluene-treated cells containing glycerol-inactivated holoDD were prepared by incubation at 37 °C for 20 min of a mixture consisting of toluene-treated cells containing 0.5 unit of DD, 30 mM potassium phosphate buffer (pH 8.0), 200 mM glycerol, 50 mM KCl, and 15 μM AdoCbl in a total volume of 1 mL. The in situ reactivation assay was carried out by incubation at 37 °C for appropriate time periods of a mixture consisting of toluene-treated cells containing 0.005 unit of glycerol-inactivated holoDD, 25 mM potassium phosphate buffer (pH 8.0), 0.1 M 1,2-propanediol, 50 mM KCl, 15 μM AdoCbl, and appropriate concentrations of ATP and $MgCl_2$ in a total volume of 1 mL. The reaction was terminated by adding 0.05 M potassium citrate buffer (pH 3.6), and the amount of propionaldehyde formed was determined by the 3-methyl-2-benzothiazolinone hydrazone method as described previously.[30]

Toluene-treated cells containing DD·CN-Cbl complex were prepared by the incubation at 37 °C for 20 min of a mixture consisting of toluene-treated cells containing 0.005 unit of DD, 30 mM potassium phosphate buffer (pH 8.0), 140 mM 1,2-propanediol, 70 mM KCl, and 20 μM CN-Cbl in a total volume of 0.7 mL. For the in situ activation assay, 15 μM AdoCbl and appropriate concentrations of ATP and $MgCl_2$ were added to a total volume of 1 mL. After incubation at 37 °C for an appropriate time period, the reaction was terminated by adding 0.05 M potassium citrate buffer (pH 3.6), and the amount of propionaldehyde formed was determined as described above.

Enzyme, Protein, and in Vitro Reactivation and Activation Assays. The amount of aldehydic products formed by DD was determined by the 3-methyl-2-benzothiazolinone hydrazone method.[30] One unit of the enzyme is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde per minute at 37 °C. The protein concentrations of purified DD and DD-R were determined by measuring the absorbance at 280 nm and using the method of Gill and von Hippel,[31] as described previously.[17]

The in vitro reactivation assay of DD-R with glycerol-inactivated holoDD as the substrate and the in vitro activation assay of DD-R with the DD·CN-Cbl complex as the substrate were performed as described previously.[17] Glycerol-inactivated holoDD and the DD·CN-Cbl complex used for these assays were prepared as described previously.[17]

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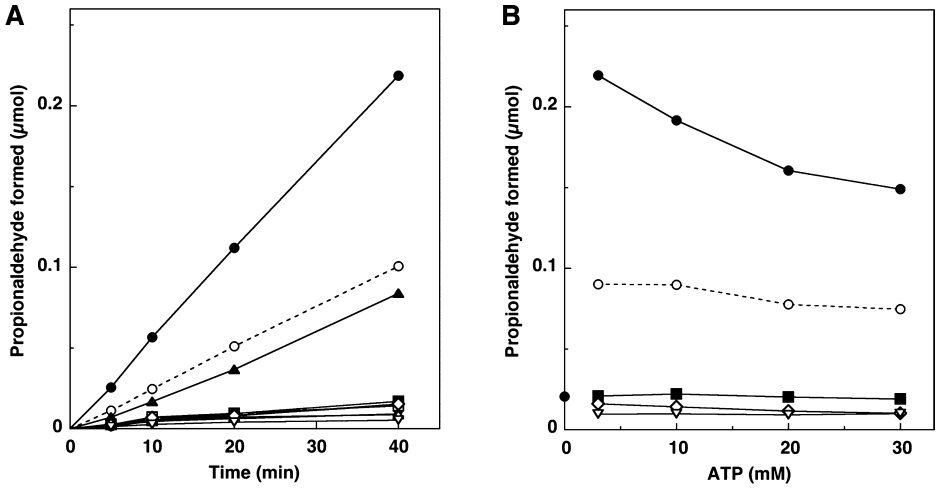

Figure 3. In situ reactivation and activation by mutant DD-Rs. Experimental procedures are described in the text. (A) In situ reactivation of glycerol-inactivated holoDD by wild-type and mutant DD-Rs: (●) wild-type; (○) wild-type (−ATP , $-Mg^{2+}$); (■) Dα8N; (◇) Dα413N; (▽) Gα556A/Gα557A; (▼) Tα105A; (□) Dα166A; (△) Dα183A; (▲) Eβ31Q. (B) In situ reactivation of glycerol-inactivated holoDD at a higher concentration of ATP by DD-Rs mutated at the nucleotide-binding site: (●) wild-type; (■) Dα8N; (◇) Dα413N; (▽) Gα556A/Gα557A. Propionaldehyde formed by non-inactivated holoDD in cells coexpressing DD and wild-type DD-R is indicated by open circles with dashed line.

PAGE Analyses. Expression of mutant DD-Rs and DDs in *E. coli* JM109 cells harboring mutated pCXV(5b-6) and pUSI2E(DD) or mutated pUSI2ENd(DD) was confirmed by SDS-PAGE analysis of the extracts from toluene-treated cells using Laemmli's sample buffer.[32] The formation of the DD·DD-R complexes was analyzed by PAGE under nondenaturing conditions, as described previously.[23] Protein was stained with Coomassie Brilliant Blue R-250.

Other Analytical Procedures. The ATP-hydrolyzing activity of mutant DD-Rs was assayed by release of [^{32}P]P_i from [γ-^{32}P]ATP in the absence of DD, as described previously.[23]

The extent of apoenzyme formation from the DD·CN-Cbl complex was estimated by spectral measurements after incubation of the complex with mutant DD-Rs in the presence and absence of nucleotide and Mg^{2+} followed by ultrafiltration with a Microcon YM-10 filter (Millipore, Billerica, MA), as described previously.[18]

Inhibition of DD by mutant DD-Rs was also determined as described previously.[18] DD was preincubated at 37 °C for the indicated time period with the mutant DD-R, and then the DD activity was measured in the presence and absence of added ATP.

RESULTS AND DISCUSSION

Residues Mutated at the Nucleotide-Binding and Metal Ion-Binding Sites. When the amino acid sequences of the α subunits of DD-R, GD-R, and EAL-R were compared with those of Hsp70 family molecular chaperones, it was found that none show overall sequence similarity but all share three regions consisting of the 15−18 amino acid residues that show high fragmentary similarities with those of the ATPase domain of *E. coli* DnaK and other Hsp70 family chaperones (Figure 2B).[1,21] These conserved regions constitute parts of three loops in their ADP-binding sites (Figure 2C).[26] To gain insight into the nucleotide-switch mechanism, the importance of these loops for reactivase function was examined by mutations of the conserved residues in each loop, that is, with the Dα8N, Dα413N, and Gα556A/Gα557A mutants.

Another functional site of DD-R that might be important for reactivase function is the metal ion-binding site in the interface between the β subunit and the swiveling domain of the α subunit. Our previous data demonstrated that subunit swapping takes place upon formation of the DD·DD-R complex.[23] Ca^{2+} is bound in the nucleotide-free form of DD-R with five ligands, including four amino acid residues (Aspα166, Aspα183, Thrα105, and Gluβ31) and a coordinated water molecule (Figure 2D).[26] These four residues are conserved in GD-R as well. This Ca^{2+} is substituted by Mg^{2+} in the ADP-bound form upon incubation with ADP and Mg^{2+}.[26] The importance of metal ion coordination for reactivase function was also examined using the Dα166A, Dα183A, Tα105A, and Eβ31Q mutants constructed.

Determination of the DD-R Activities of the Mutants by in Situ (Re)activation Assays. To confirm the expression of DD and mutant DD-R genes, toluene-treated *E. coli* cells harboring both pUSI2E(DD) (an expression plasmid for DD[33]) and mutated pCXV(5b-6) (an expression plasmid for DD-R[16]) were analyzed by SDS-PAGE using 7% polyacrylamide gel. A very intense band corresponding to the DD α subunit and a thin band corresponding to the DD-R α subunit were observed at 60K and 64K, respectively, irrespective of the sites of mutations (data not shown). This is similar to the result with the coexpression of wild-type DD-R and DD[34] and indicates that DD is expressed on a high level whereas DD-R mutants are expressed on only low levels in the coexpressing cells.

The DD-R activities of the mutants were measured by in situ assays using toluene-treated cells. In these cells, the macromolecular constituents are retained intracellularly but the permeability barrier for small molecules is destroyed.[14] As shown in Figure 3A, glycerol-inactivated holoDD formed in toluene-treated cells (in situ) was rapidly reactivated when wild-type DD-R was coexpressed in the cells. Among the mutant DD-Rs tested, only the Eβ31Q mutant of DD-R was about 40% as active as wild-type DD-R when coexpressed with DD. This is consistent with our previous finding that ∼17% activity was observed in *E. coli* cells coexpressing DdrA (the α subunit of DD-R) alone with DD.[16] This probably occurs because the same DD·DD-R complex is formed from DD and wild-type DD-R or its Eβ31Q mutant by subunit swapping. When other DD-Rs mutated at the metal ion-binding site, such

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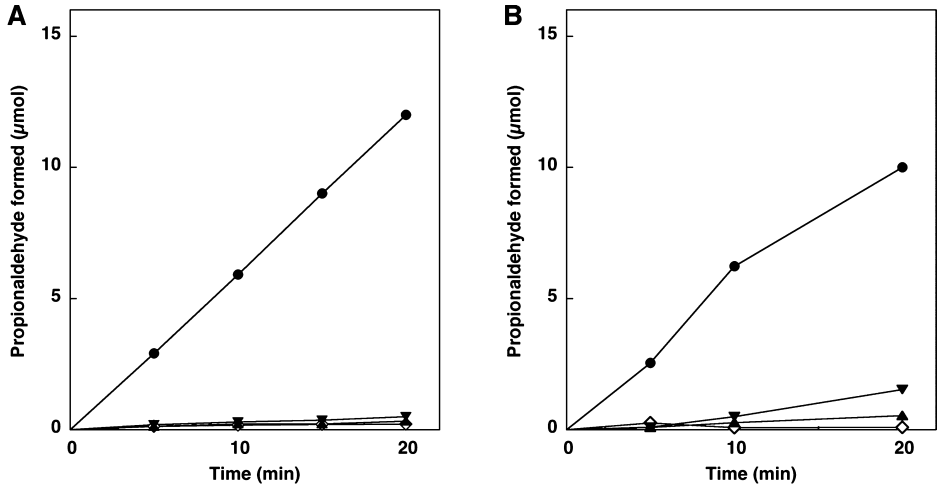

Figure 4. In vitro reactivation and activation by purified Dα8N and Dα413N mutants. (A) Glycerol-inactivated holoDD (1.4 units) or (B) DD·CN-Cbl complex (2.1 units) was incubated at 37 °C for the indicated time periods with DD-R (75 μg) in 0.03 M potassium phosphate buffer (pH 8.0) containing 20 μM AdoCbl and 1.2 M 1,2-propanediol in the presence of ATP and $MgCl_2$ (24 mM each) in a total volume of 50 μL. The reaction was terminated by adding 50 μL of 0.1 M potassium citrate buffer (pH 3.6). After removal of precipitate by centrifugation, the reaction mixture was appropriately diluted, and the amount of propionaldehyde formed was determined as described in the text. (A) In vitro reactivation of glycerol-inactivated holoDD by mutant DD-Rs. (B) In vitro activation of the DD·CN-Cbl complex by mutant DD-Rs. (●) Wild-type; (▲) Dα8N; (▼) Dα413N; (◇) without DD-R.

as Tα105A, Dα166A, and Dα183A, were coexpressed with DD, reactivation was almost not observed (Figure 3A). Essentially the same results as in Figure 3A were obtained with these mutants even when the Ca^{2+} concentration was increased up to 10 mM and when in situ activation of the DD·CN-Cbl complex was measured (data not shown). It was thus concluded that coordination of Thrα105, Aspα166, and Aspα183 to the metal ion is essential for the reactivase function of DD-R.

Coexpression of DD-Rs mutated at the nucleotide-binding site, such as Dα8N and Dα413N, was also essentially not effective for reactivation (Figure 3A). Their activities were also measured by in situ reactivation and activation assays with varying concentrations of ATP up to 30 mM (Figure 3B). However, neither Dα8N, Dα413N, nor Gα556A/Gα557A was effective as a reactivase at any ATP concentration tested. Similar results were obtained with these mutants when the in situ activation of the DD·CN-Cbl complex was measured instead of the in situ reactivation of glycerol-inactivated holoDD (data not shown). Hence, it was also concluded that these conserved residues of the three loops interacting with the pyrophosphate moiety of ADP are essential for reactivase function.

Purification and in Vitro (Re)activation Assays of DD-Rs Mutated at the Nucleotide-Binding Site. Among DD-Rs mutated at the nucleotide-binding site, the Dα8N and Dα413N mutants could be purified to homogeneity by the same procedure as described for wild-type DD-R. Their DD-R activities were determined by in vitro (re)activation assays with purified proteins. As shown in Figure 4, both mutants were almost not effective as reactivases in the reactivation of glycerol-inactivated holoDD and in the activation of the inactive DD·CN-Cbl complex. These results are consistent with the data obtained by the in situ assays and indicate that Aspα8 and Aspα413, conserved residues among reactivases and Hsp70 family chaperones, play important roles in manifestation of the reactivase function.

ATPase Activities of DD-Rs Mutated at the Nucleotide-Binding Site. The ATP-hydrolyzing activity of the purified DD-R mutants was determined using [γ-^{32}P]ATP. The data in Table 2 indicate that the ATPase activity was lowered by the Dα8N and Dα413N mutations to about 14% and 64% that of wild-type DD-R, respectively. It should be noted that the DD-R activities of the Dα8N and Dα413N mutants were <5% and <12%, respectively. It is therefore likely that the inactivity of the Dα8N mutant is due to its loss of ATPase activity, whereas the inactivity of the Dα413N mutant is not correlated with its ATPase activity. On the basis of its position in the X-ray structure, it seems reasonable that Aspα8 is involved in or assists the hydrolysis of ATP. This is consistent with the findings that Asp10 of human Hsp70 and rat heat shock cognate protein 70 (Hsc70), corresponding to Aspα8 of DD-R, is important for ATP hydrolysis.[35,36]

Table 2. ATPase Activities of Mutant DD-Rs

DD-R	[^{32}P]P_i formed (min^{-1})[a]
wild-type	2.2
Dα8N	0.31
Dα413N	1.4

[a]DD-Rs (31 and 62 μg) were incubated at 37 °C for 20 min with 3 mM [γ-^{32}P]ATP (~1.9 × 10^6 dpm) in 45 mM potassium phosphate buffer (pH 8.0) containing 3 mM $MgCl_2$ in a total volume of 50 μL. The reaction was terminated by adding 0.45 mL of an ice-cold suspension of 6% (w/v) charcoal in 50 mM NaH_2PO_4. After vigorous mixing for 10 min, the charcoal was removed by centrifugation, and the radioactivity in 0.2 mL of supernatant was determined by liquid scintillation counting. The ATPase activity was obtained by subtracting the radioactivity of a minus DD-R control.

CN-Cbl-Releasing Activity of DD-Rs Mutated at the Nucleotide-Binding Site. The direct function of DD-R is to release damaged cofactors (i.e., adenine-lacking cobalamins) from inactivated holoDD and inactive cobalamin·DD complexes, forming apoenzyme.[18] Therefore, this function of mutant DD-Rs was estimated spectroscopically by incubation of the DD·CN-Cbl complex (a model of inactivated holoDD) with mutant DD-Rs followed by ultrafiltration to remove unbound cobalamin. In the case of wild-type DD-R, enzyme-bound CN-Cbl was almost completely released during incubation in

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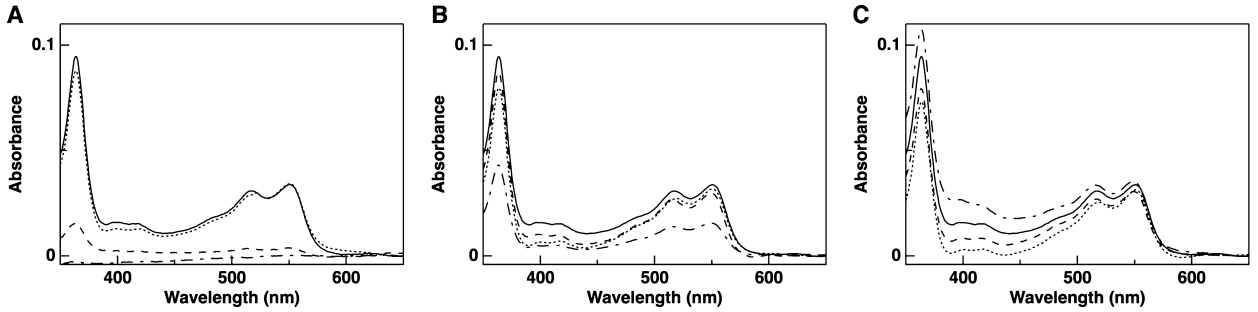

Figure 5. Ability of Dα8N and Dα413N mutants to release CN-Cbl from the DD·CN-Cbl complex. To the DD·CN-Cbl complex (4.4 units) was added 0.14 mg of DD-R in the absence or presence of ATP or ADP plus $MgCl_2$ (16 mM each) in 35 mM potassium phosphate buffer (pH 8.0) in a total volume of 90 μL. The mixtures were incubated at 37 °C for 1.5 h and then filtered on a Microcon YM-10 microconcentrator to remove unbound cobalamins. The protein fraction was washed twice with 90 μL of 35 mM potassium phosphate buffer (pH 8.0) containing 0.6% 1,2-propanediol and 0.3% Brij35 with and without ATP or ADP plus $MgCl_2$ (16 mM each). The spectrum of the fraction was measured and corrected with a minus CN-Cbl control. (A) Wild-type DD-R. (B) Dα8N. (C) Dα413N. (solid line) Control (−DD-R, none); (dotted line) DD-R (none); (dashed line) DD-R (+ATP); (dot-dashed line) DD-R (+ADP).

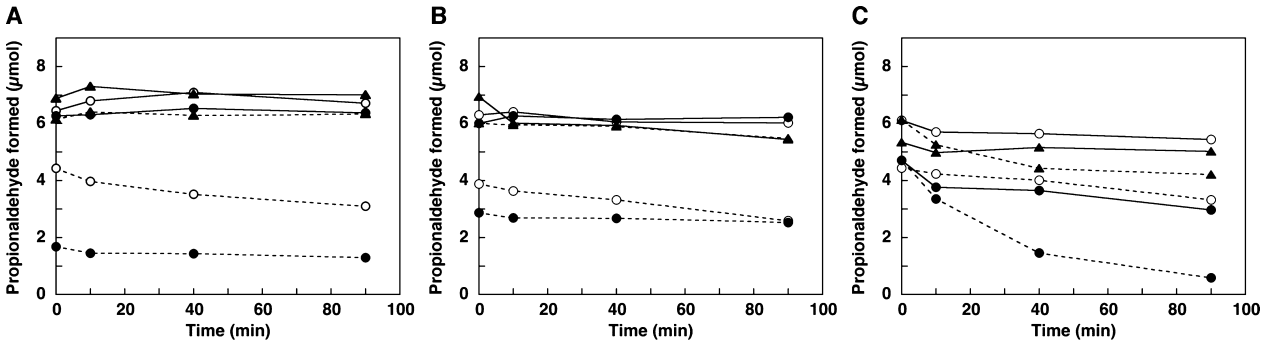

Figure 6. Inhibition of DD by preincubation with the Dα8N and Dα413N mutants in the presence and absence of nucleotide. Apoenzyme (0.74 unit) was incubated with 23 μg of DD-Rs in the absence or presence of ADP or ATP plus $MgCl_2$ (16 mM each) in 40 mM potassium phosphate buffer (pH 8.0) containing 0.8 M 1,2-propanediol in a total volume of 15 μL. The mixtures were incubated at 37 °C for the indicated time periods. AdoCbl (30 μM) was then added with (solid lines) or without (dashed lines) ATP and $MgCl_2$ (10 mM each) in a total volume of 25 μL. After 10 min at 37 °C, the reaction was terminated by adding an equal volume of 0.1 M potassium citrate buffer (pH 3.6). The resultant mixture was appropriately diluted, and the amount of propionaldehyde was determined. (A) Wild-type DD-R. (B) Dα8N. (C) Dα413N. (○) None; (●) +ADP; (▲) +ATP.

the presence of ATP or ADP (Figure 5A). When the Dα413N mutant was used instead of wild-type DD-R, CN-Cbl was not released at all during the incubation in the presence of either ATP or ADP (Figure 5C). Incubation with the Dα8N mutant in the presence of ATP did not bring about the release of CN-Cbl at all, but incubation in the presence of ADP caused about 50% release of bound CN-Cbl (Figure 5B). It is thus evident that the inactivity of DD-Rs mutated at the nucleotide-binding site can be explained by their loss of releasing activity toward CN-Cbl, an adenine-lacking cobalamin, in the presence of ATP. The nucleotide-switch mechanism of DD-R for cobalamin release seems to be lost completely in the Dα413N mutant and partially in the Dα8N mutant.

Inhibition of DD by DD-Rs Mutated at the Nucleotide-Binding Site. Tight complex formation between DD and DD-R accompanying subunit swapping leads to the release of damaged cofactors and CN-Cbl from inactivated holoDD and the DD·CN-Cbl complex, respectively.[18,23] Since DD·DD-R complexes are catalytically inactive even in the presence of AdoCbl, the complex-forming abilities of mutant DD-Rs were estimated from the inhibition of DD by DD-Rs. As shown in Figure 6A, wild-type DD-R strongly inhibited DD upon preincubation with apoDD in the presence of ADP, but the inhibition was reversed in the assay with added ATP. This occurs because ATP substitutes ADP in the nucleotide-binding site and converts DD-R to its low-affinity form for DD. In the case of the Dα8N mutant, preincubation with apoDD in the presence of ADP or even in the absence of nucleotide brought about strong inhibition of DD, and the inhibition was reversed by the addition of ATP (Figure 6B). In contrast, the preincubation of apoDD with the Dα413N mutant in the presence of ADP caused very strong inhibition in a time-dependent manner, and the inhibition was only partially reversed by the addition of ATP. These results indicate that the DD·DD-R complex of this mutant is not completely dissociable in the presence of ATP. It seems likely that the nucleotide-switch mechanism of DD-R for complex formation with DD does not perfectly operate with the Dα413N mutant but works with the Dα8N mutant.

Ability of DD-Rs Mutated at the Nucleotide-Binding Site to Form Complexes with DD. The formation of DD·DD-R complexes was directly analyzed by native PAGE. In the presence of ADP, both the Dα8N and Dα413N mutants formed complexes with wild-type apoDD (Figure 7A). When the DD·CN-Cbl complex was used instead of apoDD, the bands of the DD·DD-R complexes of the Dα8N and Dα413N mutants were much thinner than those with wild-type DD-R, although the wild type formed DD·DD-R complexes from the DD·CN-Cbl complex. This is consistent with the other results described above, that is, these mutants are inactive as reactivases

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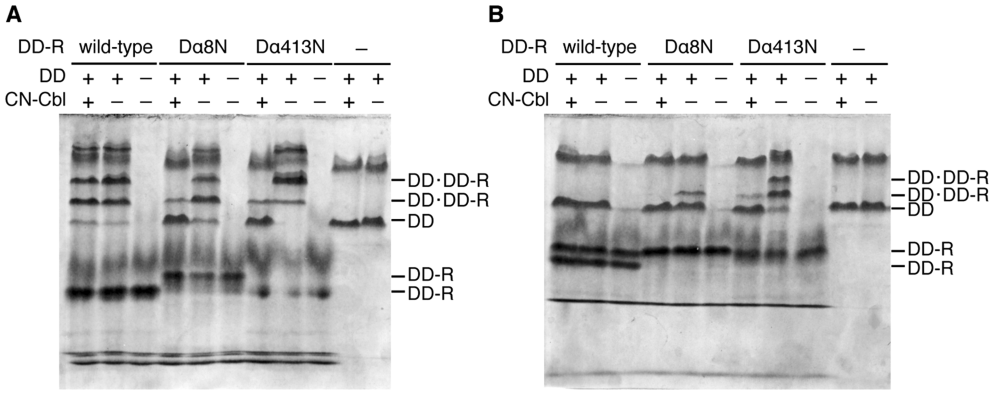

Figure 7. PAGE analysis of the abilities of the Dα8N and Dα413N mutants to form complexes with DD in the presence of nucleotide. To 0.23 unit of apoDD and DD·CN-Cbl complex was added 7.1 μg of DD-R in the presence of (A) ADP or (B) ATP plus $MgCl_2$ (5 mM each) in 8 mM potassium phosphate buffer (pH 8.0) containing 4 mM dithiothreitol in a total volume of 7.6 μL. After 1.5 h at 37 °C, the mixtures were subjected to PAGE (5.5% gel) under nondenaturing conditions in the presence of 0.1 M 1,2-propanediol, 5 mM dithiothreitol, and ADP or ATP plus $MgCl_2$ (1 mM each) in the gel using an electrode buffer containing 0.1 M 1,2-propanediol and ADP or ATP plus magnesium acetate (1 mM each). (A) +ADP. (B) +ATP. DD, DD-R, and DD·DD-R complexes are marked on the right of the gels.

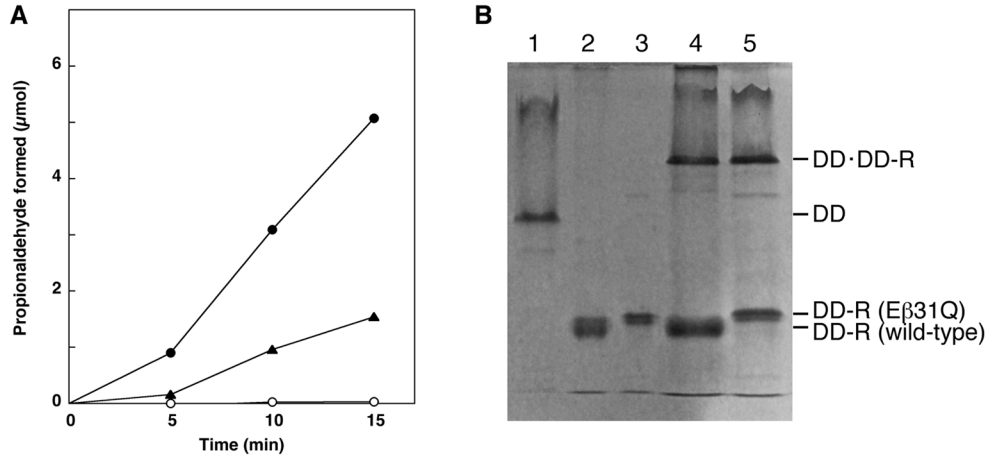

Figure 8. In vitro activation of DD·CN-Cbl complex by the purified Eβ31Q mutant and its ability to form complexes with DD. The experimental procedures were similar to those described in the captions of Figures 4 and 7. (A) In vitro activation of DD·CN-Cbl complex by wild-type DD-R (●) and Eβ31Q mutant (▲). The amount of propionaldehyde formed without DD-R is indicated by open circles. (B) PAGE analysis of the abilities of wild-type DD-R and the Eβ31Q mutant to form complexes with DD: lane 1, wild-type DD; lane 2, wild-type DD-R; lane 3, Eβ31Q DD-R; lane 4, DD + wild-type DD-R; lane 5, DD + Eβ31Q DD-R. DD, DD-R, and the DD·DD-R complexes are marked on the right of the gel.

and cannot release CN-Cbl from the DD·CN-Cbl complex but inhibit DD when incubated with apoDD in the presence of ADP. It is surprising that the Dα413N mutant formed complexes with apoDD even in the presence of ATP while the wild type did not form complex at all in the presence of ATP (Figure 7B). Complex formation with apoDD in the presence of ATP was also observed with the Dα8N mutant, but to a lesser extent. These results also indicate that the nucleotide-switch mechanism does not work with the Dα413N mutant and works partially with the Dα8N mutant, suggesting that Aspα413 is involved in the nucleotide switch. This conclusion is consistent with the fact that Asp199 of bovine Hsc70, corresponding to Aspα413 of DD-R, is involved in the coupling of ATP binding to the conformational change.[37] Aspα8 may also be involved in the switch, but only partially.

DD-Rs Mutated at the Metal Ion-Binding Site and Their Activities as Reactivases. The X-ray structure of DD-R revealed that Gluβ31 in the Glu cluster region (Gluβ30/Gluβ31/Gluβ32) is directly coordinated to the metal ion (Ca^{2+} in the nucleotide-free form and Mg^{2+} in the ADP-bound form).[26] In GD-R, this sequence is conserved in the β subunit as Gluβ30/Gluβ31/Glnβ32.[16,26] To gain insight into the role of this region, the Eβ31Q mutant was constructed and coexpressed with DD. The Eβ31Q mutant was purified to homogeneity by the same procedure as described for wild-type DD-R. Purified Eβ31Q provided both α and β subunits upon SDS-PAGE (data not shown), indicating that the α and β subunits assemble even without coordination of Gluβ31 to the metal ion. In the in vitro activation assay using the DD·CN-Cbl complex as the substrate, this mutant was about 30% as active as wild-type DD-R (Figure 8A). Figure 8B shows that it forms a tight complex with DD when incubated with apoDD. Since the DD-R β subunit dissociates upon complex formation by subunit swapping, the DD·DD-R complex formed from this mutant would be the same as that from wild-type DD-R. This is the reason that this mutant DD-R retains partial activity. It is thus likely that the β subunit of DD-R is not essential for reactivation itself but plays some role in efficient subunit swapping and/or stabilization of the α subunit by masking it from unnecessary complex formation with DD. Such a function of the β subunit might be impaired by the loss of coordination to the metal ion in the Eβ31Q mutant.

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DD Mutated at the Corresponding Glu Cluster Site of the β Subunit. The X-ray structure of DD indicates that the Gluβ96/Gluβ97/Gluβ98 residues of DD occupy the position corresponding to the Glu cluster region (Gluβ30/Gluβ31/Gluβ32) of DD-R.[26,33,38] This sequence is conserved in the β subunit of GD-R as Gluβ30/Gluβ31/Glnβ32.[16,19] In GD, this sequence is conserved in the β subunit as Gluβ63/Gluβ64/Gluβ65.[16,39] If the DD-R β subunit is displaced by DD β in subunit swapping, Gluβ97 of DD may be coordinated to the metal ion instead of Gluβ31 in the interface of the DD-R α and DD β subunits. To know the importance of this region for the reactivation of inactivated DD, the Eβ97Q mutant and the Eβ96Q/Eβ97Q/Eβ98Q triple mutant of DD were constructed and coexpressed with wild-type DD-R. When glycerol-inactivated holoDD and the DD·CN-Cbl complexes from these mutant DDs were subjected to the in situ (re)activation assays, none of the mutants were significantly (re)activated by wild-type DD-R (data not shown).

The Eβ97Q mutant was purified to homogeneity by the same procedure as described for wild-type DD. Purified Eβ97Q showed essentially the same specific activity as wild-type DD. Like the wild type, it consisted of the α, β, and γ subunits upon SDS-PAGE, indicating that the subunit assembly is not affected by the Eβ97Q mutation. When the DD·CN-Cbl complex of this mutant was used as the substrate in the in vitro activation assay, the complex was not activated at all by wild-type DD-R (Figure 9A). As shown in Figure 9B, no DD·DD-R complex

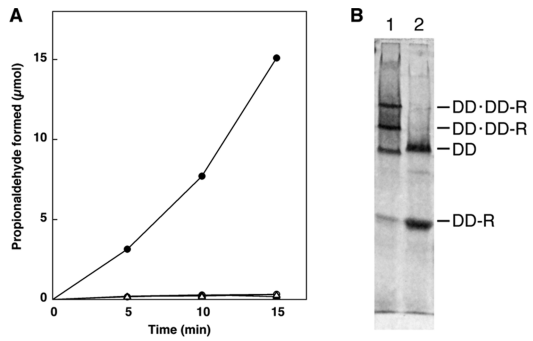

Figure 9. In vitro activation of the Eβ97Q mutant DD·CN-Cbl complex by purified wild-type DD-R and its ability to form complexes with this mutant DD. The experimental procedures were similar to those described in the captions of Figures 4 and 7. (A) In vitro activation of the complexes of wild-type DD (●) and Eβ97Q mutant (▲) with CN-Cbl by wild-type DD-R. The amount of propionaldehyde formed without DD-R is indicated by respective open symbols. (B) PAGE analysis of the abilities of wild-type DD and the Eβ97Q mutant to form complexes with wild-type DD-R: lane 1, wild-type DD + wild-type DD-R; lane 2, Eβ97Q DD + wild-type DD-R. DD, DD-R, and the DD·DD-R complexes are marked on the right of the gel.

formation was observed upon incubation of apoDD from Eβ97Q with wild-type DD-R, while wild-type apoDD formed tight complexes with DD-R. It was therefore concluded that Gluβ97 is essential for the formation of DD·DD-R complexes. The coordination of this residue to the metal ion plays an essential role for subunit swapping and, as a result, for (re)activation by DD-R.

SUMMARY

As a summary, Figure 10 depicts the mechanism of reactivation of inactivated holoDD by DD-R. It has been shown in this paper that Aspα8 and Aspα413 of DD-R participate in the ATP

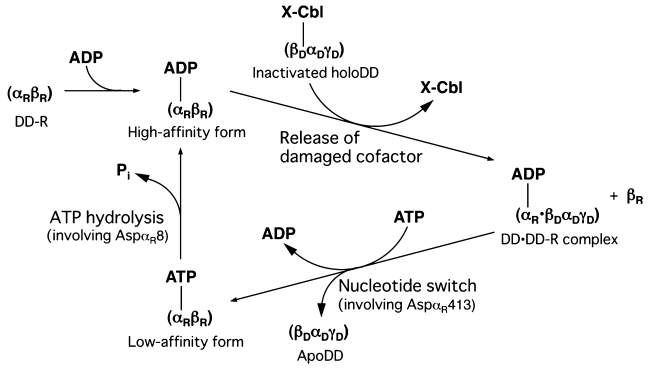

Figure 10. Possible involvement of Aspα8 and Aspα413 of DD-R in the mechanism of reactivation of inactivated holoDD. X-Cbl, a damaged cofactor or adenine-lacking cobalamin such as CN-Cbl or OH-Cbl; α_R and β_R, α and β subunits of DD-R, respectively; α_D, β_D, and γ_D, α, β, and γ subunits of DD, respectively; $(\alpha_R\beta_R)$ and $(\beta_D\alpha_D\gamma_D)$, dimeric proteins of DD-R and DD, respectively.

hydrolysis and the nucleotide-switch, respectively. Although the structure of DD-R in complex with a nonhydrolyzable ATP analogue is not available yet, the conformation of the Aspα413-containing loop might be sensitive to a bound nucleotide and important for maintaining a radical status of holoDD. Recently, Banerjee and co-workers reported that a mobile loop in MeaB determines its chaperone function for AdoCbl-dependent methylmalonyl-CoA mutase.[40] Reactivases for AdoCbl-dependent enzymes and Hsp70 chaperones can be considered as high-specificity and low-specificity molecular chaperones, respectively, that have evolved from a common ancestor protein. A common ATP/ADP-switch mechanism was suggested from the finding that the roles of conserved Asp residues of the loops located in their nucleotide-binding sites are similar. This seems reasonable because the reactivases and Hsp70 chaperones are proteins whose interactions with other proteins or polypeptides are altered by nucleotide-induced conformational changes. Gluβ31, a Mg^{2+}-coordinating residue of DD-R, is not essential for reactivation. In contrast, Gluβ97 of DD is absolutely required for DD·DD-R complex formation and (re)activation, indicating that its coordination to Mg^{2+} in DD-R is necessary for subunit swapping and thus for complex formation.

AUTHOR INFORMATION

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Notes

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ABBREVIATIONS:

AdoCbl, adenosylcobalamin or coenzyme B_{12}; buffer A, 0.05 M potassium phosphate buffer (pH 8.0); CN-Cbl, cyanocobalamin; DD, diol dehydratase; DD-R, diol dehydratase-reactivase;

EAL, Ethanolamine ammonia-lyase; EAL-R, Ethanolamine ammonia-lyase-reactivase; GD, glycerol dehydratase; GD-R, glycerol dehydratase-reactivase; OH-Cbl, hydroxocobalamin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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