Characterization and Mechanism of Action of a Reactivating Factor for Adenosylcobalamin-dependent Glycerol Dehydratase*

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Adenosylcobalamin-dependent glycerol dehydratase undergoes mechanism-based inactivation by its physiological substrate glycerol. We identified two genes (gdrAB) of Klebsiella pneumoniae for a glycerol dehydratase-reactivating factor (Tobimatsu, T., Kajiura, H., Yunoki, M., Azuma, M., and Toraya, T. (1999) J. Bacteriol. 181, 4110–4113). Recombinant GdrA and GdrB proteins formed a tight complex of (GdrA),2(GdrB),2, which is a putative reactivating factor. The purified factor reactivated the glycerol-inactivated and O2-inactivated glycerol dehydratases as well as activated the enzyme-cyanocobalamin complex in situ in the presence of ATP, Mg2+, and adenosylcobalamin. The factor mediated the exchange of the enzyme-bound, adenine-lacking cobalamins for free, adenine-containing cobalamins in the presence of ATP and Mg2+ through intermediate formation of apoenzyme. The factor showed extremely low ATP-hydrolyzing activity and formed a tight complex with apoenzyme in the presence of ADP. Incubation of the enzyme-cyanocobalamin complex with the reactivating factor in the presence of ADP brought about release of the enzyme-bound cobalamin. The resulting tight inactive complex of apoenzyme with the factor dissociated upon incubation with ATP, forming functional apoenzyme and a low affinity form of factor. Thus, it was established that the reactivation of the inactivated holoenzymes takes place in two steps: ADP-dependent cobalamin release and ATP-dependent dissociation of the apoenzyme-factor complex. We propose that the glycerol dehydratase-reactivating factor is a molecular chaperone that participates in reactivation of the inactivated enzymes.

Glycerol dehydratase (EC 4.2.1.30) is an enzyme that catalyzes adenosylcobalamin (AdoCbl)1 (coenzyme B12)-dependent conversion of glycerol, 1,2-propanediol, and 1,2-ethanediol to the corresponding aldehydes (1–3). The enzyme is involved in the formation of β-hydroxypropionaldehyde, an electron acceptor, in the fermentation of glycerol through the dihydroxyacetone pathway (4–6). Although the enzyme is produced by some genera of the family Enterobacteriaceae, such as Klebsiella and Citrobacter, and other bacteria when they are grown anaerobically in a medium containing glycerol (1–4, 7), this enzyme undergoes mechanism-based inactivation by glycerol during catalysis (3, 8, 9). The glycerol-inactivated enzyme in permeabilized cells (in situ) of Klebsiella pneumoniae is rapidly reactivated by exchange of the modified coenzyme for intact AdoCbl in the presence of ATP and Mg2+ (or Mn2+) (10). The complex of enzyme with CN-Cbl is also activated in situ under the same conditions (11). Recently, we identified two open reading frames in the vicinity of the glycerol dehydratase genes (12) as the genes encoding reactivating factor for glycerol dehydratase and designated them gdrAB (13). The products of the genes formed a tight complex that was considered a putative reactivating factor.

Diol dehydratase (EC 4.2.1.28), an isofunctional enzyme, is inducibly formed when certain bacteria are grown in a medium containing 1,2-propanediol (3). This enzyme involved mainly in the utilization of 1,2-propanediol by Enterobacteriaceae and other bacteria (3, 14) also undergoes mechanism-based inactivation by glycerol (15, 16). A reactivating factor for diol dehydratase (DdrA/DdrB complex (17)) of Klebsiella oxytoca has been well characterized (18), and the mechanism of action established in vitro (19). It is a molecular chaperon-like factor that mediates ATP-dependent release of the tightly bound, modified coenzyme from the inactivated holoenzyme. The reactivation takes place in two steps (i.e. ADP-dependent cobalamin release and ATP-dependent dissociation of apodiol dehydratase-reactivating factor complex (19)).

The amino acid sequences of GdrA and GdrB are 61 and 30% identical to those of DdrA and DdrB, respectively (17). Although functional similarity of these reactivating factors was suggested from these sequence similarities, the reactivating factor for glycerol dehydratase has not yet been investigated in vitro. In the present paper, we report the purification, characterization, and mechanism of action of the recombinant glycerol dehydratase-reactivating factor. During the preparation of this manuscript, a paper has appeared that reports characterization of a similar factor of Citrobacter freundii (20), but the mechanism of its function still remains obscure.

EXPERIMENTAL PROCEDURES

Materials—Crystalline AdoCbl was a gift from Eisai Co., Ltd. (Tokyo, Japan). CN-Cbl was obtained from Glaxo Wellcome. AdePeCbl was prepared as described before (21). [γ-32P]ATP and [57Co]-CN-Cbl (specific activity, 240 μCi/μg) were obtained from PerkinElmer Life Sciences and ICN Pharmaceuticals, respectively. Glycerol dehydratase was purified from Escherichia coli JM109 carrying pUS12E(GD) (12) by Sepharose CL-6B and hydroxyapatite column chromatography. Glycerol dehydratase with a specific activity of more than 50 units/mg of protein was used in this study. All other chemicals and the enzymes used for construction of plasmids were commercial products of the highest grade available and were used without further purification.

Bacterial Strain, Plasmids, and Culture Conditions—E. coli BL21(DE3) was used as a host. The expression plasmid for the glycerol
dehydratase-reactivating factor was constructed as follows. The plasmid pCXVgdrBgdra (13) was digested completely with BglII and partially with NdeI. The resulting 2.2-kilobase pair DNA fragment was inserted into the NdeI-BamHI region of pET21a to produce pET(gdrBgdra). E. coli BL21(DE3) carrying plasmid pETgdrBgdra was transformed in Terrific Broth containing 50 μg/ml ampicillin at 30 °C. When the culture reached an A600 of ~1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a concentration of 1 mM. The E. coli cells were cultivated overnight at 17 °C, harvested, and stored at −80 °C until use.

**Purification of the Recombinant Reactivating Factor**—About 15 g of wet cells were suspended in 150 ml of 0.05 M potassium phosphate buffer (pH 8) containing 2 mM EDTA and 2 mM phenylmethanesulfonyl fluoride and disrupted by sonication. To the cell extract obtained after centrifugation at 27,200 × g for 30 min, ammonium sulfate was added to 5% saturation. After removal of a precipitate by centrifugation, ammonium sulfate was added to 25% saturation. The precipitate was collected, dissolved in 15 ml of 0.05 M potassium phosphate buffer (pH 8) containing 2 mM EDTA and 2 mM phenylmethanesulfonyl fluoride, and dialyzed against 5 mM potassium phosphate buffer (pH 8) containing 0.5 mM EDTA. Dialysate was applied onto a Sepharose CL-6B column (bed volume, 500 ml) that was equilibrated with 1.2-propanediol. The column was developed with the same buffer, and the fractions containing GdrA and GdrB were collected and concentrated by ultrafiltration through a Centriplus-10 concentrator (Amicon) and stored at −80 °C.

**Assays of Enzyme and Reactivating Factor**—The amount of propionaldehyde formed from 1,2-propanediol was determined by the 3-methyl-2-benzothiazolinone hydrazone method (22). One unit of glyceraldehyde formed from 1,2-propanediol was determined by the method of Davis (25) or under denaturing conditions as described by Laemmli (26). Nondenaturing PAGE of glycerol dehydratase was performed under nondenaturing conditions as described by Davis (25) or under denaturing conditions as described by Davis (25) or under denaturing conditions as described by Davis (25) or under denaturing conditions as described by Davis (25). Non-denaturing PAGE of glyceraldehyde dehydratase was performed in the presence of 0.1 M 1,2-propanediol to prevent its subunits from dissociation (27). In some experiments, ATP or ADP was also added with MgCl2 (1 mM each) and KCl (2 mM) to gels and electrode buffer. Protein was stained with Coomassie Brilliant Blue G-250. The concentrations of purified glyceraldehyde dehydratase and its reactivating factor were determined by measuring the absorbance at 280 nm. The molar absorption coefficient at 280 nm calculated by the method of Gill and von Hippel (24) for the reactivating factor was 86,500 M−1 cm−1.

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**ATPase Activity**—The products formed by the hydrolysis of ATP with the reactivating factor were analyzed by TLC on a poly(ethyleneimine)-cellulose plate (Merck) with 2 M formic acid containing 0.5 M LiCl as a solvent system (28), as described before (19). ATP-hydrolyzing activity of the reactivating factor was assayed by the release of [γ32P]ATP, as described before (19) by a modification of the method of Schnell and Abrams (29). An appropriate amount of the factor was incubated at 37 °C for 30 min with 3 mM [γ32P]ATP (−3.1 × 108 dpm) in 20 mM potassium phosphate buffer (pH 8) containing 3 mM MgCl2, in a total volume of 100 μl. After termination of the reaction by adding 0.9 ml of ice-cold suspension of 6% (w/v) charcoal in 50 mM NaH2PO4 and mixing vigorously for 10 min, the charcoal was removed by centrifugation. The amount of radioactivity in 0.5 ml of the supernatant was determined by liquid scintillation counting, and ATPase activity was obtained by subtracting the radioactivity of a minus reactivating factor control.

**RESULTS**

**Purification and Subunit Structure of the Recombinant Reactivating Factor**—The Mw of the two proteins (64,000 and 12,000) overexpressed in E. coli BL21(DE3) carrying pET(gdrBgdrA) corresponded to the predicted molecular weights of GdrA (63,594) and GdrB (11,994), respectively. They were co-purified through ammonium sulfate fractionation and Sepharose CL-6B column chromatography (Fig. 1A) and mi-

**FIG. 1. Purification of the GdrA and GdrB proteins.** Fractions at each purification step were subjected to SDS-PAGE (denaturing) on 12% polyacrylamide gel (A) and PAGE (nondenaturing) on 7% polyacrylamide gel (B). Proteins were stained with Coomassie Brilliant Blue. Molecular weight markers were SDS-7 (Sigma) (A). Positions of GdrA, GdrB, and their complexes (indicated with arrowheads to the right of the gels. BPP, bromphenol blue).

**Activation of the Enzyme-Cobalamin Complex by the Reactivating Factor**—Activation of the enzyme-cobalamin complex by the reactivating factor was assayed with 1,2-propanediol as substrate by its capability of activating the inactive enzyme-CN-Cbl complex. This activity was well correlated with its capability of reactivating the glycerol-inactivated holoenzyme.

**Protein Assay**—Protein concentration of crude enzyme and reactivating factor was determined by the method of Lowry et al. (23) with crystalline bovine serum albumin as a standard. The concentrations of purified glycerol dehydratase and its reactivating factor were determined by measuring the absorbance at 280 nm. The molar absorption coefficient at 280 nm calculated by the method of Giun and von Hippel (24) for the reactivating factor was 86,500 M−1 cm−1.

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**Evidence for Cobalamin Exchange**—The absolute requirement of both reactivation of glycerol-inactivated and O2-inactivated holoenzymes and activation of the enzyme-CN-Cbl complex for free AdoCbl strongly suggested that the reactivation and the activation take place by exchange of the enzyme-
bound, modified coenzyme and CN-Cbl, respectively, for free intact AdoCbl. CN-Cbl and AdePeCbl were used as models of the modified and intact coenzymes, respectively (18). When the enzyme-CN-Cbl complex was incubated with or without the reactivating factor in the presence of AdePeCbl, ATP, and Mg2+, followed by dialysis to remove unbound cobalamins, the spectrum of the dialysate indicated that the enzyme-bound CN-Cbl was replaced by AdePeCbl in the presence of factor (Fig. 3A). This exchange did not occur without the factor. The reverse was not the case even in the presence of factor, ATP, and Mg2+ (Fig. 3B). Thus, it was suggested that the reactivating factor mediate the exchange of the enzyme-bound, adenine-lacking cobalamin for free, adenine-containing cobalamin.

To examine the possibility that only an upper ligand undergoes exchange under the conditions, the enzyme-[57Co]CN-Cbl complex was incubated with and without the factor in the presence of AdePeCbl, ATP, and Mg2+ (Fig. 3B). Thus, it was suggested that the reactivating factor mediates the exchange of the enzyme-bound, adenine-lacking cobalamin for free, adenine-containing cobalamin.

To examine the possibility that only an upper ligand undergoes exchange under the conditions, the enzyme-[57Co]CN-Cbl complex was incubated with and without the factor in the presence of AdePeCbl, ATP, and Mg2+, followed by ultrafiltration to remove unbound cobalamins. One of the typical results is shown in Table I. The [57Co] radioactivity bound to glycerol dehydratase was almost completely lost in the presence of factor, ATP, and Mg2+, whereas the amount of radioactivity corresponding to more than 1 mol of CN-Cbl/mol of enzyme was retained in the protein fraction in the absence of factor. In the presence of ATP, a half of [57Co]CN-Cbl bound to enzyme was lost by the factor. This result, together with the stoichiometric observation, indicated that the entire molecule of enzyme-bound CN-Cbl underwent the exchange for free AdePeCbl or release in the presence of reactivating factor. It is evident that ADP is more effective than ATP for this exchange or release.

Evidence for Intermediary Formation of Apoglycerol Dehydratase in the Reactivation—Glycerol-inactivated holoenzyme

and the enzyme-aqCbl5'-deoxyadenosine complex were incubated with the reactivating factor in the presence of ATP, Mg2+, and K2SO3 and then dialyzed to remove unbound cobal-
dehydratase and the GdrA and GdrB proteins were detected.

The molar ratio of glycerol dehydratase to reactivating factor was calculated to be 1:3 in this experiment.

ATP-hydrolyzing Activity of the Reactivating Factor—When ATP was incubated with the reactivating factor, time-dependent decrease of ATP and formation of ADP were observed by TLC (data not shown). Release of 32P into the dialysate from the glycerol-dehydratase by PAGE. Enzyme-AdePeCbl (E-AdePeCbl) and enzyme-CN-Cbl (E-CN-Cbl) complexes were prepared by incubation of apoenzyme (apoE) (2.25 units) with 50 µM AdePeCbl and CN-Cbl, respectively, at 37 °C for 30 min in 22.5 µl of 33 mM potassium phosphate buffer (pH 8). To 3.5 µl of each complex and apoenzyme were added 15 µg of ADP to reactivating factor to a volume of 6.5 µl, and the mixtures were incubated at 37 °C for 10 min. They were further incubated at 37 °C for 10 min in the absence (A) and presence of 21 mM ATP plus 21 mM MgCl2 (B) or 21 mM ADP plus 21 mM MgCl2 (C) in 35 mM potassium phosphate buffer (pH 8) in a total volume of 7.5 µl. The mixtures were subjected to PAGE (5% gel) under nondeaturating conditions in the absence (A) and presence of 1 mM ATP plus 1 mM MgCl2 (B) or 1 mM ADP plus 1 mM MgCl2 (C). Positions of glycerol dehydratase (E), reactivating factor (RF), and their complexes (E-RF) are indicated with arrowheads to the right of the gels. BPB, bromphenol blue.

Release of Enzyme-bound Cobalamin upon Enzyme-Factor Complex Formation—When the enzyme-CN-Cbl and enzyme-AdePeCbl complexes were incubated with ATP, only a small amount of the enzyme-factor complex was formed from the enzyme-AdePeCbl complex, a “nonactivable” complex, whereas a large amount of the enzyme-factor complex was formed from the enzyme-CN-Cbl complex, an “activable” complex (Fig. 5C). The second band of the complex was observed in these cases. It may have an assembly different from the first one, although their exact molar compositions were not determined.

Inhibition of Apoenzyme by the Reactivating Factor and Reversal by ATP—The effect of enzyme-factor complex formation on catalytic activity was investigated with added coenzyme. When apoglycerol dehydratase was incubated with the reactivating factor in the presence of ADP, it lost enzyme activity in

ATP-hydrolyzing activity (kcat) of the factor during glycerol dehydratase (reactivating condition) was 0.9 min⁻¹, which was significantly lower than that during 1,2-propanediol dehydratase (essentially nonreactivating condition) (1.6 min⁻¹) or in the absence of AdoCbl (1.5 min⁻¹). The molar ratio of glycerol dehydratase to reactivating factor was ~1:3 in this experiment. When the rate of ATP hydrolysis by the factor was measured with various ratios of the enzyme to the factor, ATP-hydrolyzing activity decreased until the molar ratio became to ~2:1 (Fig. 4). It was thus suggested that the ATPase activity of the reactivating factor is inhibited by its binding to glycerol dehydratase.

Complex Formation between Glycerol Dehydratase and the Reactivating Factor—Complex formation between glycerol dehydratase and its reactivating factor was analyzed by nondeaturating PAG (Fig. 5). When apoenzyme was incubated with the factor in the presence of ADP, a new major band appeared (Fig. 5C). When this band was developed in the second dimension by SDS-PAGE, all of the α, β, and γ subunits of glycerol dehydratase and the GdrA and GdrB proteins were detected (data not shown), indicating that this band is a complex between the enzyme and the factor. Formation of the apoenzyme-factor complex was much less in the absence of nucleotides (Fig. 5A) and not detectable at all in the presence of ATP (Fig. 5B). Therefore, the ADP- and ATP-bound reactivating factors are high and low affinity forms for the apoenzyme, respectively. When the factor was incubated with enzyme-cobalamin complexes, the enzyme-factor complex was not formed at all from either enzyme-AdePeCbl or enzyme-CN-Cbl complex in the presence of ATP (Fig. 5B) or in its absence (Fig. 5A). In the presence of ADP, only a small amount of the enzyme-factor complex was formed from the enzyme-AdePeCbl complex, a “nonactivable” complex, whereas a large amount of the enzyme-factor complex was formed from the enzyme-CN-Cbl complex, an “activable” complex (Fig. 5C). The second band of the complex was observed in these cases. It may have an assembly different from the first one, although their exact molar compositions were not determined.

FIG. 5. Analyses of complex formation between the reactivating factor and glycerol dehydratase by PAG. Enzyme-AdePeCbl (E-AdePeCbl) and enzyme-CN-Cbl (E-CN-Cbl) complexes were prepared by incubation of apoenzyme (apoE) (2.25 units) with 50 µM AdePeCbl and CN-Cbl, respectively, at 37 °C for 30 min in 22.5 µl of 33 mM potassium phosphate buffer (pH 8). To 3.5 µl of each complex and apoenzyme were added 15 µg of ADP to reactivating factor to a volume of 6.5 µl, and the mixtures were incubated at 37 °C for 10 min. They were further incubated at 37 °C for 10 min in the absence (A) and presence of 21 mM ATP plus 21 mM MgCl2 (B) or 21 mM ADP plus 21 mM MgCl2 (C). Positions of glycerol dehydratase (E), reactivating factor (RF), and their complexes (E-RF) are indicated with arrowheads to the right of the gels. BPB, bromphenol blue.

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resulting mixtures was added no reactivating factor (thick solid line). The protein fraction was washed twice with 90 mM phosphate buffer (pH 8) for 1.5 h and then ultrafiltered, as described in the legend to Table I.

Enzyme complexes were prepared by incubation of apoenzyme (21 units) with 15 μM CN-Cbl and AdoPecBl, respectively, at 37 °C for 30 min in 140 μl of 45 mM potassium phosphate buffer (pH 8). To 30 μl of the resulting mixtures was added no reactivating factor (thick solid line) or 0.14 μM of reactivating factor in the absence (thin solid line) and presence of 16 mM ATP plus 16 mM MgCl₂ (broken line) or 16 mM ADP plus 16 mM MgCl₂ (dotted line) in 35 mM potassium phosphate buffer (pH 8) in a total volume of 90 μl. The mixtures were incubated at 37 °C for 1.5 h and then ultrafiltrated, as described in the legend to Table I. The protein fraction was washed twice with 90 μl of 35 mM potassium phosphate buffer (pH 8) with and without 16 mM ATP plus 16 mM MgCl₂ or 16 mM ADP plus 16 mM MgCl₂. The spectrum of the protein fraction was measured and corrected with a minus cobalamin control.

a time-dependent manner (Fig. 7C). ADP alone did not inhibit the activity (data not shown). The inhibition was observed in the absence of ADP as well but to a lesser degree (Fig. 7B). In contrast, inhibition was not observed at all in the presence of ATP. It is likely that the extent of inhibition of enzyme activity by the factor depends on formation of the enzyme-factor complex. Thus, it was concluded that the apoenzyme-factor complex is unable to be reconstituted into active holoenzyme with added AdoCbl. When ATP was added together with AdoCbl, it completely reversed the inhibition (Fig. 7), probably by facilitating dissociation of the inactive apoenzyme-factor complex into free apoenzyme and the factor. Such an effect of ATP on the dissociation of the complex was confirmed by non-denaturing PAGE (data not shown) and seems reasonable, because the ATP-bound reactivating factor is the low affinity form for glyceraldehyde.

DISCUSSION

Glycerol dehydratase as well as diol dehydratase undergoes rapid inactivation by glycerol during catalysis (3, 8, 9, 15, 16), although glycerol is a physiological substrate for these enzymes (3–6). This enigma was solved by our discovery of the reactivation systems in the bacteria that produce these enzymes (10, 11). The subunit structure, function, and mechanism of action of the putative reactivating factor was unraveled with diol dehydratase for the first time (18, 19) and now with glycerol dehydratase. Recently, we obtained evidence for the presence of a specific reactivating factor for ethanolamine ammonia-lyase as well.2 Thus, it seems likely that other AdoCbl-dependent enzymes also have their own reactivating factors. This prediction would be quite reasonable, because all of the AdoCbl-dependent enzymes catalyze reactions by a radical mechanism and therefore tend to undergo inactivation during catalysis or even in the absence of substrate (32).

The reactivating factor for diol dehydratase was shown to cross-reactivate the glycerol dehydratase in permeabilized E. coli cells (33). This fact was supported by the finding that the reactivating factor for glycerol dehydratase was very similar to that for diol dehydratase in the subunit structure and the functional role. However, it should be noted that the glycerol dehydratase-reactivating factor was not capable of cross-reactivating the inactivated holozyme of diol dehydratase (20, 33). This indicates that there must be differences between them as well.

The reactivating factor showed low but distinct ATPase activity. Although the hydrolysis of ATP was absolutely required for reactivation of the inactivated holozymes by the reactivating factor (10), the ATP hydrolysis was not directly linked to the reactivation of the glycerol-inactivated holozyme. Such a phenomenon is seen with diol dehydratase-reactivating factor (19) as well and is not unusual in nature (34, 35). The inhibition of ATPase activity of the reactivating factor by binding to apoenzyme under reactivating conditions was observed here for the first time. This may be suitable for cellular economy of energy.

Fig. 8A shows the two-step mechanism that we propose for the reactivation of glycerol-inactivated hologlycerol dehydratase. The O₂-inactivated holozyme and the enzyme-CN-Cbl complex can be (re)activated by the same mechanism. First, the factor-bound ATP is hydrolyzed to ADP, which induces the conformational change of the factor from a low affinity form to a high affinity form. Complexation of the ADP form with the reactivating factor and reversal by ATP.

2 K. Mori and T. Toraya, manuscript in preparation.
Glycerol Dehydratase-reactivating Factor

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