Adenosylcobalamin-dependent diol dehydratase of Klebsiella oxytoca undergoes suicide inactivation by glycerol, a physiological substrate. The coenzyme is modified through irreversible cleavage of its cobalt-carbon bond, resulting in inactivation of the enzyme by tight binding of the modified coenzyme to the active site. Recombinant DdrA and DdrB proteins of K. oxytoca were co-purified to homogeneity from cell-free extracts of Escherichia coli overexpressing the ddrAB genes. They existed as a tight complex, i.e. a putative reactivating factor, with an apparent molecular weight of 150,000. The factor consists of equimolar amounts of the two subunits with $M_r$ of 64,000 (A) and 14,000 (B), encoded by the $ddrA$ and $ddrB$ genes, respectively. Therefore, its subunit structure is most likely $A_2B_2$. The factor not only reactivated glycerol-inactivated and $\gamma$-inactivated holoenzymes but also activated enzyme-cyanocobalamin complex in the presence of free adenosylcobalamin, ATP, and $Mg^{2+}$. The reactivating factor mediated ATP-dependent exchange of the enzyme-bound cyanocobalamin for free 5-adeninylpentylcobalamin in the presence of ATP and $Mg^{2+}$, but the reverse was not the case. Thus, it can be concluded that the inactivated holoenzyme becomes reactivated by exchange of the enzyme-bound, adenine-lacking cobalamin for free adenosylcobalamin, an adenine-containing cobalamin.

Diol dehydratase (propane-1,2-diol hydro-lyase, EC 4.2.1.28) is an enzyme that catalyzes the adenosylcobalamin (AdoCbl)$^1$-dependent conversion of 1,2-propanediol, glycerol, and 1,2-ethanediol to the corresponding aldehydes (1, 2). This enzyme is inducibly formed by some genera of Enterobacteriaceae, such as Klebsiella and Citrobacter, and other bacteria when they are grown anaerobically in medium containing 1,2-propanediol (3, 4). When some of these bacteria are grown anaerobically on glycerol, they form glycerol dehydratase as well (3, 4). Diol and glycerol dehydratases are important for producing essential electron acceptors in the fermentation of 1,2-propanediol and glycerol, respectively (5–9). Klebsiella oxytoca ATCC 8724 is defective in glycerol dehydratase (10, 11), but the reverse was not the case. Thus, it can be concluded that the inactivated holoenzyme becomes reactivated by exchange of the enzyme-bound, adenine-lacking cobalamin for free adenosylcobalamin, an adenine-containing cobalamin.

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Materials—Crystalline CN-Cbl was obtained from Glaxo Research Laboratories, Ltd., Greenford, UK. AdoCbl was a gift from Eissai Co., Ltd., Tokyo, Japan. AdePeCbl was synthesized by the published procedure (21). Recombinant diol dehydratase was purified to homogeneity from an overexpressing Escherichia coli JM109 harboring expression plasmid pUSI2E(DD) (22).

Construction of Expression Plasmids—We have constructed expression plasmid pCXV(6/5b) for the ddrAB genes using vector pCXV (20). Because copy numbers of plasmids containing replication origin of p15A, like pCXV are less than those of plasmids containing replication origin of pBR322, like pUSI2E (23), (10–12 and 15–20 copies/cell, respectively (24)), we transferred the genes from pCXV(6/5b) to another expression vector, pUSI2END. The 6.6-kilobase HindIII-BglII fragment containing the tac promoter sequence and the ddrA and ddrB genes was excised from pCXV(6/5b) and ligated to the 5-kilobase HindIII-BglII fragment of pUSI2END(DD) to construct pUSI2END(DD). pUSI2END(DD) was a derivative of pUSI2E(DD) (23), an expression plasmid for the diol dehydratase genes. The unnecessary NdeI site on the vector region of pUSI2E(DD) was eliminated in pUSI2E(DD). E. coli JM109 was used as a host for expression plasmid pUSI2END(6/5b).

Cultivation of Overexpressing E. coli Strain—Recombinant DdrA and DdrB proteins of K. oxytoca were purified to homogeneity from E. coli JM109 harboring expression plasmid pUSI2END(6/5b) that was aero-
The capability of reactivating factor to reactivate glycerol-inactivated diol dehydratase reaction was determined by the 3-methyl-2-benzothiazolinone hydrazone method (18).

The elution of proteins was monitored by absorbance at 280 nm. The amount of enzyme activity that catalyzes the formation of propionaldehyde was measured by the 3-methyl-2-benzothiazolinone hydrazone method (18). 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 for determining the amount of propionaldehyde formed by the 3-methyl-2-benzothiazolinone hydrazone method (18).

The capability of reactivating factor to reactivate glycerol-inactivated or O2-inactivated holodiol dehydratases by the reactivating factor. Glycerol-inactivated holoenzyme was prepared by incubation of apoenzyme (331 units) with 50 μM AdoCbl at 37 °C for 30 min in 2.5 ml of 0.05 M potassium phosphate buffer (pH 8) containing 30% glycerol, followed by dialysis at 4 °C for 48 h against 800 volumes of 0.05 M potassium phosphate buffer (pH 8). Glyceraldehyde-inactivated holoenzyme (1.5 units) or O2-inactivated holoenzyme (1.5 units) was incubated at 37 °C for the indicated time periods with (●) and without (▲) 47 μg of the reactivating factor in 0.02 M potassium phosphate buffer (pH 8) containing 21 μM AdoCbl and 1.2 M 1,2-propanediol in the presence (●) and absence (○) of 24 mM ATP/24 mM MgCl2 in a total volume of 50 μl. Time course of 1,2-propanediol dehydration with non-inactivated enzyme was measured as a control (△). 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 for determining the amount of propionaldehyde formed by the 3-methyl-2-benzothiazolinone hydrazone method (18).

The elution of proteins was monitored by absorbance at 280 nm. The amount of enzyme activity that catalyzes the formation of propionaldehyde was measured by the 3-methyl-2-benzothiazolinone hydrazone method (18). 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 for determining the amount of propionaldehyde formed by the 3-methyl-2-benzothiazolinone hydrazone method (18).

Protein Assays—During purification of diol dehydratase and the ddrA and ddrB gene products, protein concentrations were routinely estimated by the method of Lowry et al. (25) with crystalline bovine serum albumin as a standard. The concentration of purified diol dehydratase was determined by measuring the absorbance at 280 nm. The molar absorption coefficient at 280 nm (ε280) calculated by the method of Gill and von Hippel (26) for diol dehydratase from its deduced amino acid compositions (23) and subunit structure (22) was 120,500 M−1 cm−1. Based on the molecular weight predicted, ε280 was calculated to be 5.81 for diol dehydratase.

PAGE—PAGE was performed under non-denaturing conditions essentially as described by Davis (27), except that 5 mM dithiothreitol was added in the gel, or under denaturing conditions as described by Laemmli (28). Protein was stained with Coomassie Brilliant Blue R-250. Densitometric analysis of gels was performed with a Printgraph AE-6911CX system (ATTO, Tokyo, Japan) and the NIH-Image program, Version 1.61 (National Institutes of Health).

Edman Sequencing of the Subunits—A purified preparation of the complex of the DdrA and DdrB proteins was separated into the subunits (A and B polypeptides, respectively) by SDS-PAGE (15.0%) and electrophoretically transferred to a polyvinylidene difluoride membrane (Applied Biosystems). Protein bands were visualized with Coomassie Brilliant Blue R-250, excised, and analyzed for NH2-terminal amino acid sequences on an Applied BioSystems 491 protein sequencer.

Molecular Weight Determination by Gel Filtration—The molecular weight of the complex (putative reactivating factor) was determined by gel filtration on Superose 6 column (HR10/30) using a FPLC system (Amersham Pharmacia Biotech). The purified factor and molecular weight marker proteins were applied to the column, which was equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM KCl and developed with the same buffer at a flow rate of 0.4 ml/min. The elution of proteins was monitored by A280.

Enzyme Assays—The amount of aldehydic products formed by diol dehydratase reaction was determined by the 3-methyl-2-benzothiazolinone hydrazone method (29). One unit of diol dehydratase is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde/min at 37 °C. Reactivation of the inactivated holodiol dehydratase by the reactivating factor was determined using 1,2-propanediol or glycerol as a substrate in the presence of 21 μM AdoCbl, 24 mM ATP, and 24 mM MgCl2.

The capability of reactivating factor to reactivate glycerol-inactivated or O2-inactivated holodiol dehydratase was assayed in vitro using 1,2-propanediol as substrate. The capability of it to activate the inactive enzyme-CN-Cbl complex was also assayed in vitro because these two capabilities in situ were shown to be well correlated (18).
**RESULTS**

**Purification of Recombinant DdrA and DdrB Proteins—**The *ddrA* and *ddrB* gene products were co-purified from extracts of overexpressing *E. coli*. All operations were performed at 0–4 °C. Throughout the purification steps, purity of the proteins in each fraction was analyzed by SDS-PAGE.

About 10 g of wet cells grown at 30 °C were suspended in 50 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 2 mM EDTA and 2 mM PMSF and disrupted by sonication for 10 min at 240 W with a Kajo Corp. TA-5287 ultrasonic destruction system (Japan). After centrifugation at 27,200 × g for 30 min, the supernatant was collected. The precipitate was washed with 60 ml of the same buffer, and the washing was combined with the supernatant (cell-free extract).

Solid ammonium sulfate was added to the cell-free extract to 15% saturation. After centrifugation, solid ammonium sulfate was added again to the supernatant to 35% saturation. The precipitate was dissolved in 20 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 2 mM EDTA and 2 mM PMSF and dialyzed for 1 day against 2 liters of 5 mM potassium phosphate buffer (pH 8.0) containing 0.5 mM EDTA with one buffer change.

The dialysate was applied to a DEAE-cellulose column (bed volume, 80 ml) which was equilibrated with 2 mM potassium phosphate buffer (pH 8.0) and then developed with the same buffer, and the fractions containing the DdrA and DdrB proteins were pooled, concentrated to about 5 ml by ultrafiltration through a Diaflo PM-10 membrane and Centriplus (Amicon). The concentrated solution was loaded onto a Sephadex G-200 column (bed volume, 150 ml) which was equilibrated with the 50 mM potassium phosphate buffer (pH 8.0). The column was developed successively with 280 ml of the same buffer and with 200 ml of 6 mM potassium phosphate buffer (pH 8.0) and then with 200 ml of the same buffer containing 0.5 mM MgCl₂. The DdrA and DdrB proteins-containing fractions were pooled and concentrated to about 1.7 ml by ultrafiltration through a Diaflo PM-10 membrane and Centriplus (Amicon).

The concentrated solution was loaded onto a Sephadex G-200 column (bed volume, 150 ml) which was equilibrated with the 50 mM potassium phosphate buffer (pH 8.0). The column was developed with the same buffer, and the fractions containing the DdrA and DdrB proteins were pooled, concentrated to about 5 ml by ultrafiltration through a Centriplus, and stored at −80 °C.

**Purity, Molecular Weight, and Subunit Structure of the Purified Reactivating Factor—**It is evident that the two bands with *M₅* of 64,000 and 14,000 (designated A and B polypeptides, respectively) were overexpressed in *E. coli* JM109 carrying pUS12End(6/5b) (Fig. 1A). Both of these bands were progressively enriched upon purification. When the purified preparation was electrophoresed under non-denaturing conditions in the presence of dithiothreitol, it migrated as a single band (Fig. 1B).

Therefore, it was clear that the two polypeptides were co-purified as a tight complex. The predicted molecular weights of the DdrA and DdrB proteins are 64,266 and 13,620, respectively (20), it was highly suggested that the A and B polypeptides are the products of these genes. The NH₂-terminal 10-amino acid sequences of the A and B polypeptides determined by Edman sequencing were MRYIAGIDIG and MNGNHSAPAI, respectively. These sequences agreed com-
plete with those deduced from the nucleotide sequences of the ddrA and ddrB genes (20). Thus, the A and B polypeptides were undoubtedly identified as the ddrA and ddrB gene products, respectively. These results indicate that the DdrA and DdrB proteins are co-purified and exist as a tight complex under non-denaturing conditions. This complex was considered as a putative reactivating factor.

To determine the subunit composition of the factor, the complex purified to homogeneity was separated into subunits by SDS-PAGE in a 15% gel and stained with Coomassie Brilliant Blue R-250. Densitometric analysis of the bands, together with molecular weights of the subunits predicted from the amino acid composition, indicated that the molar ratio of the A and B polypeptides in the complex was approximately 1:1.1. The apparent molecular weight of the complex determined by FPLC with a calibrated Superose 6 column (HR 10/30) was approximately 150,000 (data not shown). By taking the predicted molecular weights of the subunits into consideration, it can be concluded that the subunit structure of the putative reactivating factor is most likely A2B2.

The molar absorption coefficient at 280 nm (εmax,280) for the complex (reactivating factor), calculated by the method of Gill and von Hippel (26) from its amino acid compositions and subunit structure, was 58,140 M⁻¹ cm⁻¹, ε15,280 for the reactivating factor was calculated to be 3.73 on the basis of the predicted molecular weight.

**In Vitro Reactivation of Inactivated Holoenzymes by the Reactivating Factor**—The capability of the putative reactivating factor to reanimate the glycerol-inactivated holodiol dehydratase was examined *in vitro* using 1,2-propanediol as substrate. As illustrated in Fig. 2A, *in vitro* reactivation of the glycerol-inactivated holoenzyme by the purified factor in the presence of AdoCbl, ATP, and Mg²⁺ was observed for the first time. The reactivation did not take place at all without the factor or with the factor but in the absence of ATP/Mg²⁺. Free AdoCbl was also absolutely required for the reactivation (data not shown). Thus, it is evident that the factor actually functions as a reactivating factor for glycerol-inactivated diol dehydratase. The product formed increased exponentially at the initial stage of reaction and then almost linearly with time of incubation. By comparison of the maximum slope with the control, the extent of reactivation under the conditions employed was estimated to be approximately 64%.

Diol dehydratase holoenzyme is known to undergo inactivation by O₂ in the absence of substrate (30). This inactivation is considered because of reaction of the activated Co—C bond of the enzyme-bound coenzyme with O₂. Fig. 2B shows that the O₂-inactivated holoenzyme also undergoes reactivation by the factor in the presence of AdoCbl, ATP, and Mg²⁺. The extent of reactivation increased with time of incubation and reached at least 71% at 20 min. Again, the reactivation was strictly dependent on the factor and on ATP/Mg²⁺ and free AdoCbl.

**In Vitro Activation of the Enzyme-CN-Cbl Complex by the Reactivating Factor**—The capability of the reactivating factor to activate the inactive complex of diol dehydratase with CN-Cbl was also examined *in vitro* because the inactive enzyme-CN-Cbl complex can be considered as a model of the inactivated holoenzyme. Fig. 3A indicates that the enzyme-CN-Cbl complex is rapidly activated by the factor in the presence of AdoCbl, ATP, and Mg²⁺. This activation by the factor also required ATP/Mg²⁺ (Fig. 3A) in addition to free AdoCbl (data not shown). Approximately 76% of the enzyme-CN-Cbl complex underwent activation by 20 min of incubation under the conditions. As shown in Fig. 3B, the extent of reactivation was dependent on a molar ratio of the factor to the enzyme-CN-Cbl complex. From the double-reciprocal plot, the concentration of the factor giving half-maximal activation of 1.2 μM enzyme-CN-Cbl complex was calculated to be 3.5 μM.

**Direct Evidence for Cobalamin Exchange**—The reactivating factor, free AdoCbl, ATP, and Mg²⁺ were absolutely required for both reactivation of the glycerol-inactivated holoenzyme and activation of the enzyme-CN-Cbl complex. ADP was not able to replace ATP. From the absolute requirement for free AdoCbl, it was strongly suggested that the reactivation of the glycerol-inactivated holoenzyme and activation of the enzyme-CN-Cbl complex occurs by exchange of the enzyme-bound, modified coenzyme and CN-Cbl, respectively, for free intact AdoCbl. We have previously reported that the *in situ* reactivation of the glycerol-inactivated holoenzyme or the *in situ* activation of inactive cobalamin-enzyme complexes takes place by exchange of enzyme-bound cobalamins for AdoCbl (13, 18).

To examine whether the reactivating factor mediates such exchange, the enzyme-CN-Cbl complex was subjected to incubation with and without the reactivating factor in the presence of AdePeCbl, ATP, and Mg²⁺, followed by dialysis to remove unbound cobalamins. AdePeCbl, an inactive analog of AdoCbl containing the adenine ring in the upper axial ligand, was used...
Dehydration was determined with and without the reactivating exchange. To test this possibility, the time course of glycerol that the reactivating factor may mediate only a single cide inactivation at a significant rate, there remains a possibil-

tivity restored. Because 1,2-propanediol does not cause sui-
tained with 1,2-propanediol as substrate for measuring enzyme activity restored. Because 1,2-propanediol does not cause sui-

The data shown in Figs. 2 and 3 were ob-

Fig. 5. Reactivation of glycerol-inactivated holodiol dehydratase by the reactivating factor during dehydration of glycerol. A, diol dehydratase apoenzyme (1.5 units) was incubated at 37 °C for 2, 5, and 10 min with 130 μM AdoCbl in 40 μl of 0.03 M potassium phosphate buffer (pH 8) containing 1.5 M glycerol. At 10 min of incubation (arrow), 47 (▲), 23 or 0 (●) μg of the reactivating factor was added to a reaction mixture with (▲, ●) and without (□, □) 0.1 M ATP/24 mM MgCl2 in a total volume of 50 μl. The mixture was further incubated at 37 °C for the indicated time periods. In one experiment, the reactivating factor, ATP, and MgCl2 were added to the reaction mixture at the start of the reaction (●). The amount of β-hydroxypropionaldehyde formed was determined after appropriate dilution. B, diol dehydratase apoenzyme (1.5 units) was incubated with 100 μM AdoCbl at 37 °C for 4 h together with and without the indicated amount of reactivating factor in 0.02 M potassium phosphate buffer (pH 8) containing 1.2 M glycerol in the presence (●) and absence (○) of 24 mM ATP/24 mM MgCl2. The amount of β-hydroxypropionaldehyde formed was determined after appropriate dilution. The numbers of reactivation per diol dehydratase (▲) and per reactivating factor (○) are shown in the inset.

Instead of AdoCbl itself because the complex of diol dehydratase with AdoCbl (regular holoenzyme) is catalytically active and rather susceptible to inactivation even in the presence of substrate (31). As depicted in Fig. 4A, the spectrum of the dialysate indicates that the enzyme-bound CN-Cbl was re- placed by AdePeCbl with the reactivating factor. This exchange never occurred without the factor. Thus, it is evident that the reactivating factor mediates the exchange of the enzyme-bound CN-Cbl for free AdePeCbl. In contrast, the reverse was not the case. That is, the replacement of the enzyme-bound AdePeCbl by free CN-Cbl did not occur at all even with the factor in the presence of ATP and Mg2+ (Fig. 4B). Thus, it is quite likely that the reactivating factor mediates the exchange of enzyme-bound, adenine-lacking cobalamins for free, adenine-containing cobalamins. Because the coenzyme loses the adenine moi- ety by irreversible cleavage of the Co—C bond in the inactivation of holoenzymes by glycerol or O2, it can therefore be concluded that the reactivating factor reactivates the inac- tivated holoenzymes or activates the enzyme-CN-Cbl complex by mediating the ATP-dependent exchange of the enzyme-bound, modified coenzyme or CN-Cbl, i.e. adenine-lacking cobalamins, for free intact AdoCbl, i.e. an adenine-containing cobalamin.

Numbers of Reactivation per Diol Dehydratase and per Re-
activating Factor—The data shown in Figs. 2 and 3 were ob-

AdoCbl-dependent enzymatic reactions are initiated by ho-

moly of the Co—C bond of the enzyme-bound coenzyme and proceed via radical mechanisms (1, 3, 4, 32–36). Such reactions are considered to need the assistance of high reactivity of a free radical. Highly reactive radical intermediates must sustain their reactivity at the active sites and become extinct in the only way destined for the reaction. Once a radical intermediate becomes extinct or stabilized by side reactions, regeneration of AdoCbl becomes impossible, resulting in irreversible modification of the coenzyme (3). This leads to inactivation of enzymes because the modified coenzymes remain tightly bound to en-

DISCUSSION

AdoCbl-dependent enzymatic reactions are initiated by ho-

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zymes and not exchangeable with free AdoCbl. Are such inac-
tivated enzymes reactivated? This would be important for cel-

lular economics of energy. The data presented in this paper
Diar Dehydratase-reactivating Factor

Diol Dehydratase-reactivating Factor (20). Therefore, such reactivating factor may be a factor of general importance for AdoCbl-dependent enzymes.

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