Characterization, Sequencing, and Expression of the Genes Encoding a Reactivating Factor for Glycerol-inactivated Adenosylcobalamin-dependent Diol Dehydratase*

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Diol dehydratase undergoes suicide inactivation by glycerol during catalysis involving irreversible cleavage of the Co-C bond of adenosylcobalamin. In permeabilized Klebsiella oxytoca and Klebsiella pneumoniae cells, the glycerol-inactivated holoenzyme or the enzyme-cyano-cobalamin complex is rapidly activated by the exchange of the inactivated coenzyme or cyanocobalamin for free adenosylcobalamin in the presence of ATP and Mg\textsuperscript{2+}(Honda, S., Toraya, T., and Fukui, S. (1980) J. Bacteriol. 143, 1458–1465; Usui, K., Honda, S., Toraya, T., and Fukui, S. (1982) J. Nutr. Sci. Vitaminol. 28, 225–236). Permeabilized Escherichia coli cells co-expressing the diol dehydratase genes with two open reading frames in the 5‘-flanking region were capable of reactivating glycerol-inactivated diol dehydratase as well as activating the enzyme-cyanocobalamin complex in situ in the presence of free adenosylcobalamin, ATP, and Mg\textsuperscript{2+}. These open reading frames, designated as \textit{ddrA} and \textit{ddrB} genes, were identified as the genes of a putative reactivating factor for inactivated diol dehydratase. The genes encoded polypeptides consisting of 610 and 125 amino acid residues with predicted molecular weights of 64,266 and 13,620, respectively. Co-expression of the open reading frame in the 5‘-flanking region was stimulatory but not obligatory for conferring the reactivating activity upon \textit{E. coli}. Thus, the product of this gene was considered not an essential component of the reactivating factor.

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Diol dehydratase (1,2-propanediol hydro-lyase, EC 4.2.1.28) catalyzes AdoCbl\textsuperscript{1}-dependent conversion of 1,2-propanediol, glycerol, and 1,2-ethanediol to the corresponding aldehydes (1, 2). The enzyme is inducibly formed by some genera of Enterobacteriaceae, such as Klebsiella and Citrobacter, and other bacteria which produce a diol dehydratase when they are grown anaerobically in a medium containing 1,2-propanediol (3, 4). The enzyme participates in the fermentation of this substrate (5, 6). When some of these bacteria are grown anaerobically on glycerol, glycerol dehydratase is induced and involved in producing an electron acceptor for the fermentation of glycerol via the dihydroxyacetone pathway (7–9). Although \textit{Klebsiella oxytoca} (formerly \textit{Klebsiella pneumoniae} and \textit{Aerobacter aerogenes}) ATCC 8724 is defective in glycerol dehydratase (10, 11), it is capable of fermenting glycerol. This is because a low level of diol dehydratase induced by glycerol substitutes for isofunctional glycerol dehydratase (2, 7, 12, 13). Both dehydratases undergo inactivation by glycerol during catalysis (2, 14–16). Inactivation by glycerol is mechanism-based and involves irreversible cleavage of the Co-C bond of AdoCbl, forming 5‘-deoxyadenosine and an alkyl-cobalamin-like species (3, 14). Irreversible inactivation of the enzyme is brought about by tight binding of the modified coenzyme (3, 14, 17). Such suicide inactivation seemed enigmatic, because glycerol is a growth substrate for \textit{K. oxytoca}. This apparent inconsistency was solved by our finding that the glycerol-inactivated enzyme in permeabilized cells (\textit{in situ}) of \textit{K. oxytoca} undergoes rapid reactivation by exchange of the modified coenzyme for intact AdoCbl in the presence of ATP and Mg\textsuperscript{2+} (or Mn\textsuperscript{2+}) (13, 18). The enzyme-CN-Cbl complex is also activated \textit{in situ} under the same conditions. Such reactivation and activation are detectable only \textit{in situ} but not \textit{in vitro}. It remained unclear whether the reactivation is caused by a specific factor, although some factor(s) necessary for the \textit{in situ} reactivation was indirectly suggested to be inducible by glycerol (13).

We have cloned and sequenced the \textit{pdd} genes encoding diol dehydratase of \textit{K. oxytoca} ATCC 8724 and obtained overexpressing \textit{Escherichia coli} strains (19). In this paper, we report characterization of the genes encoding a reactivating factor for glycerol-inactivated diol dehydratase by sequencing and co-expression with the \textit{pdd} genes using two kinds of mutually compatible expression vectors.

EXPERIMENTAL PROCEDURES

Materials—Crystalline AdoCbl was a gift from Eisai Co. Ltd. (Tokyo, Japan). CN-Cbl was obtained from Glaxo Research Laboratories (Greenford, UK). 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 Strains, Plasmids, and Culture Conditions—The genes encoding reactivating factor were isolated from plasmid pUCD11, which contains a 10.5-kb chromosomal DNA insert from \textit{K. oxytoca} (19). \textit{E. coli} HB101 and \textit{E. coli} JM109 were used as hosts, and plasmids pUSI2E (19) and pCXV (this study) used as expression vectors. Transformation of \textit{E. coli} was performed by the electroporation method of Dower et al. (20).

Recombinant strains harboring expression plasmids were aerobically grown at 37 °C in LB medium containing 1,2-propanediol (0.1%) and ampicillin (50 μg/ml) (for strains harboring expression plasmids derived from pUSI2E) or chloramphenicol (50 μg/ml) (for strains harboring expression plasmids derived from pCXV) (19). When the culture reached an \textit{A}_{600} of approximately 0.8, isopropyl-1-thio-β-D-galactopyranoside was added for induction to a concentration of 1 mM. Cells were...
harvested in the late logarithmic phase.

Preparation of Permeabilized Cells—Permeabilized cells were prepared by treatment with 1% (v/v) toluene as described previously (13), except that the treatment was performed on a small scale in 1.5-ml microtubes.

Enzyme Assay—The amount of aldehyde products formed by diol dehydratase reaction was determined by the 3-methyl-2-benzothiazoline hydrazone method (21).

SDS-PAGE—Cells were disrupted by sonication. SDS-PAGE of cell homogenates was carried out as described by Laemmli (22). Protein bands were stained with Coomassie Brilliant Blue R-250.

DNA Sequencing—Standard recombinant DNA techniques were performed as described by Sambrook et al. (23). Restriction endonucleases and the enzymes for construction of plasmids were used according to the manufacturer’s instructions.

Nucleotide Sequencing—Template single-stranded DNAs were prepared from the plasmids carrying restriction fragments and deletion mutants of pUCD11 (19). DNA sequencing was performed by the dideoxyribonucleotide chain termination method of Sanger et al. (24) using a Sequencing Pro kit (Toyobo Co., Osaka, Japan), Klengow fragment of E. coli DNA polymerase I (Life Technologies, Inc.), and Sequenase (U. S. Biochemical Corp.).

Constructions of Plasmids—The 6.8-kb HpaI-EcoRI fragment from pUCD11 and the 0.15-kb BamHI-HpaI fragment from pUSI2E(DD) were ligated with pUSI2E previously linearized with BamHI and EcoRI to construct pUSI2E(DD5). The 7.5-kb HindIII-EcoRI fragment from pUCD11 and the 0.24-kb BamHI-HindIII fragment from pUSI2E(DD) were ligated with pUSI2E previously linearized with BamHI and EcoRI to construct pUSI2E(DD5+).

A 2.3-kb DNA segment of pSTV25 (Takara Shuzo Co., Ltd., Kyoto, Japan) was amplified by PCR using Vent DNA polymerase (New England Biolabs) and oligonucleotide primers TCAACGTTTTGAGGGCA-GAATAAAATGATACATC and AGCTCGGTAGCCCGCTATAATGGAGC-GGCTTTTTTATGAGAATATTACATTATATCGTAG (the Hind-III and AvaI sites and the trpA transcriptional terminator are underlined). The PCR product was digested with HindIII and AvaI and ligated with the 3.1-kb HindIII-EcoRI fragment from pUCD11 to construct pUCD11-DD. The resulting DNA segment encoding the N-terminal region of ORF6 was amplified by PCR using Vent DNA polymerase, 5’-primer AGCATATGACAAATTCGCTGGGAGCAAAT (initiation codon GTG of ORF5a was replaced by underlined ATG), and 3’-primer ATAAAATAT-TTCGTGCTTCGGCTT (complementary to the nucleotide sequence 0.1-kb downstream of the unique SalI site). The PCR product digested with NdeI and SalI and the 2.9-kb SalI-EcoRI fragment from pUSI2E(DD5+) was ligated with the 4.4-kb NdeI-EcoRI fragment from pUCD11-DD to construct pCXXV(DD). A DNA segment encoding the C-terminal region of ORF5 was amplified by PCR using Vent DNA polymerase (Stratagene), 5’-primer TGGCATATATGGAGGACTCTG and reverse primer (complementary to the nucleotide sequence 0.2-kb upstream of the unique Csp45I site), and 3’-primer TCGATGTTCAAGAGCCCAACGCT. The PCR product digested with Csp45I and BglII and the 0.8-kb Csp45I-BglII fragment was ligated with the 5.4-kb BglII-SalI fragment from pCXXV(DD) to construct pCXXV(DD) was constructed in a similar way, except that the 5.3-kb BglII-SalI fragment from pCXXV(DD) was used. A DNA segment encoding the region of ORF6 was amplified by PCR using Pfu DNA polymerase, 5’-primer TGGCATATATGGAGGACTCTG and reverse primer (complementary to the nucleotide sequence 0.2-kb upstream of the unique Csp45I site), and 3’-primer TCGATGTTCAAGAGCCCAACGCT. The PCR product digested with Csp45I and BglII and the 0.8-kb Csp45I-BglII fragment was ligated with the 5.4-kb BglII-SalI fragment from pCXXV(DD) to construct pCXXV(DD) was constructed in a similar way, except that the

RESULTS

In Situ Reactivation of Glycerol-inactivated Diol Dehydratase in E. coli Co-expressing Genes of Diol Dehydratase and the Flanking Regions—As illustrated in Fig. 1, plasmid pUCD11 carries a 10.5-kb genomic DNA of K. oxytoca containing the pdd genes encoding diol dehydratase (ORFs 2–4) and their flanking regions (19). There exist ORF1 and ORF5, etc., with unknown functions in the 5’- and 3’-flanking regions, respectively. Recently, we found that that E. coli harboring plasmid pUCD11 was capable of reactivating glycerol-inactivated diol dehydratase in situ in the presence of free AdoCbl, ATP, and Mg2+. We have previously reported such in situ reactivation with K. oxytoca ATCC 8724 and K. pneumoniae ATCC 29585 (13). Therefore, it was strongly suggested that some protein(s) encoded by gene(s) in the flanking regions of the pdd genes are responsible for the in situ reactivation. To discover which flanking region of the diol dehydratase genes is essential for reactivation of glycerol-inactivated diol dehydratase, we constructed two expression plasmids, pUSI2E(DD5+) and pUSI2E(DD5+), which contain the 3'-flanking region from pUCD11 in addition to the pdd genes and the pdd genes plus ORF1, respectively (Fig. 2A).

As shown in Fig. 3A, deactivation of glycerol by permeabilized E. coli cells carrying pUSI2E(DD5+) with added AdoCbl was accompanied by concomitant inactivation and ceased almost completely within 3 min, as did permeabilized K. oxytoca cells (13) or diol dehydratase in vitro (2, 14). However, when ATP and Mg2+ were supplemented to the reaction mixture in addition to AdoCbl, an initial, rapid phase of glycerol dehydrolysis was followed by a slower but almost constant rate of the dehydrolysis. Furthermore, when ATP and Mg2+ were added to the mixture at 10 min after the reaction was initiated (at which time essentially all the diol dehydratase present in the reaction mixture had been inactivated by glycerol), the inactivated enzyme underwent rapid reactivation to give the same rate of dehydrolysis as that with initially added ATP and Mg2+. These characteristics of the in situ reactivation in the recombinant E. coli cells agree well with those observed with K. oxytoca and K. pneumoniae cells (13). In contrast, the in situ reactivation of
and Mg$_2^+$ underwent activation with free AdoCbl in the presence of ATP. As shown in Table I, nearly half of the diol dehydratase-dehydratase and the flanking regions.

The capability of recombinant E. coli cells to activate the inactive enzyme-CN-Cbl complex in $situ$ was also assayed using 1,2-propanediol as substrate. 1,2-Propanediol is a substrate that does not bring about significant suicide inactivation of the enzyme (1, 2, 14). It has been established before (18) that the expression of ORF1 in addition to the thick protein bands with $\gamma$ subunits of diol dehydratase are indicated with arrowheads in the right of the gels.

Toluene-treated E. coli JM109 (4 x 10$^6$ cells) carrying pUSI2E(DD5+) (A) or pUSI2E(DD) (B) was incubated at 37 °C for the indicated time with 15 $\mu$m AdoCbl in 30 mM potassium phosphate buffer (pH 8.0) containing 63 mM KCl and 0.25 mM 1,2-propanediol in a total volume of 0.8 ml. AdoCbl was then added to the mixture to a final concentration of 15 $\mu$m with and without ATP and MgCl$_2$ (3 mM each) in a total volume of 1.0 ml. The amount of propionaldehyde formed between 5 and 10 min of incubation after the addition of AdoCbl was determined.

In $situ$ activation of the diol dehydratase-CN-Cbl complex in E. coli co-expressing the genes of diol dehydratase and the flanking regions on a single expression vector

| Host/plasmid | Extent of activation$^a$ |
|--------------|-------------------------|
|              | With ATP/MgCl$_2$ | Without ATP/MgCl$_2$ |
| JM109/pUSI2E(DD5+) | 39 | 0.0 |
| JM109/pUSI2E(DD) | 0.2 | 0.0 |
| JM109/pUSI2E(1DD5+) | 66 | 0.1 |
| JM109/pUSI2E(1DD) | 0.0 | 0.0 |

$^a$ The extent of in $situ$ activation of the enzyme-CN-Cbl complex was calculated on the basis of the amount of propionaldehyde formed between 5 and 10 min of incubation by permeabilized cells preincubated without CN-Cbl.
detected in common in the homogenates of *E. coli* carrying pUSI2E(DD51) and pUSI2E(1DD51) (Fig. 2C). Thus, this seemed to be one of the candidates for product(s) of genes in the 3′-flanking region.

**Sequence Analysis of the 3′-Flanking Region That Is Essential for the in Situ Reactivation—**

Because the 3′-flanking region is essential for the in situ reactivation, the nucleotide sequences of ORF5 (ddrA gene) and ORF6 (ddrB gene) and deduced amino acid sequences of the diol dehydratase-reactivating factor are shown in Fig. 4. Nucleotides are numbered beginning with the first nucleotide of the translational initiation codon of the ORF5b. Amino acid symbols are written below the first nucleotide of the corresponding codons, and amino acids are numbered beginning with each N-terminal residue of the products of ORF5b and ORF6. The putative ribosome-binding sites (Shine-Dalgarno sequences) are underlined. Sequences putatively forming secondary structures are marked by arrows, indicating the lengths and orientation of the stems.

**Fig. 4. Nucleotide Sequences of ORF5 (ddrA gene) and ORF6 (ddrB gene) and deduced amino acid sequences of the diol dehydratase-reactivating factor.** Nucleotides are numbered beginning with the first nucleotide of the corresponding codons, and amino acids are numbered beginning with each N-terminal residue of the products of ORF5b and ORF6. The putative ribosome-binding sites (Shine-Dalgarno sequences) are underlined. Sequences putatively forming secondary structures are marked by arrows, indicating the lengths and orientation of the stems.
region of the *pdd* genes was essential for both in situ reactivation of the inactivated holoenzyme and activation of the enzyme-CN-Cbl complex, the region was subjected to nucleotide sequencing according to the strategy shown in Fig. 1. As summarized in Figs. 1 and 4, there existed two ORFs (ORF5 and ORF6) in the immediate downstream of the diol dehydratase genes in the same direction. The 3′-end of ORF5 overlapped the 5′-end of ORF6 by 8 nucleotides.

Two possible initiation codons were found in ORF5: the GTG and ATG codons that were located at 41–43 and 206–208 nucleotides downstream of the termination codon of the *pddC* gene. For convenience, ORFs starting from these GTG and ATG codons are referred to as ORF5a and ORF5b, respectively. ORF5a, ORF5b, and ORF6 encode polypeptides consisting of 665, 610, and 125 amino acid residues with predicted molecular weights of 70,517, 64,266, and 13,620, respectively. Shine-Dalgarno sequences were found 8–11 bases upstream of the putative initiation codons. Two sets of inverted repeat sequences that may form hairpin structures exist immediately downstream of this GTG codon.

**Construction of an Expression Vector That Is Compatible with *pUSI2E* in *E. coli*—**For co-expression of ORFs in the 3′-flanking region with the *pdd* genes in *E. coli*, we constructed an expression vector, pCXV. This vector possesses the *lac* repressor gene, a *tac* promoter, a ribosome binding site, the *trpA* transcriptional terminator, replication origin of p15A, and the chloramphenicol acetyltransferase gene (Fig. 5A). The *lac* repressor gene, the *tac* promoter, the ribosome binding site,
and cloning sites of pCXV are common to those of pUSI2E. Because vector pUSI2E has a replication origin of pBR322 and the β-lactamase gene (Fig. 2A) (19), pCXV and pUSI2E could be stably co-transformed to E. coli cells, and co-transformants were readily selected by resistance to both chloramphenicol and ampicillin. Copy numbers of plasmids containing replication origins of p15A and pBR322 are 10–12 and 15–20 copies/cell, respectively (23). E. coli JM109 carrying expression plasmid pCXV(DD), which contains the pdd genes downstream of the tac promoter of pCXV, produced an amount of diol dehydratase comparable with that produced by E. coli JM109 carrying pUSI2E(DD) (data not shown).

High Level Expression of ORF5 and ORF6 Using Vector pCXV in E. coli—To characterize the gene products of ORF5 and ORF6, we constructed seven expression plasmids derived from pCXV (Fig. 5A). E. coli JM109 was transformed with these plasmids, and homogenates of the recombinant E. coli strains were analyzed by SDS-PAGE (Fig. 5, B and C). E. coli harboring plasmids containing ORF5b produced a thick protein band with M<sub>r</sub> of 64,000. On the other hand, E. coli harboring plasmid carrying ORF5a produced two bands with M<sub>r</sub> of 71,000 and 64,000 (Fig. 5C). A thin protein band with M<sub>r</sub> of 64,000 was also observed in homogenates of E. coli harboring plasmids containing both the pdd genes and the 3′-flanking region (Fig. 2C). These lines of evidence indicate that the real product of ORF5 is the M<sub>r</sub> 64,000 polypeptide, namely the product of ORF5b. Therefore, it was strongly suggested that the real initiation codon of ORF5 was the ATG but not the GTG. In the numbering in Fig. 4, the first nucleotide of this translational initiation codon (ATG) of ORF5b and the first amino acid of the ORF5b product are taken as 1. E. coli cells harboring expression plasmids containing ORF6 produced a M<sub>r</sub> 14,000 polypeptide, although the band of this product was not thick. The largest amount of the ORF6 product was obtained in E. coli harboring plasmid pCXV(6/5b) that contains ORF5b and ORF6 in the reverse order (Fig. 5B). The M<sub>r</sub> 64,000 and 14,000 polypeptides partially purified were subjected to Edman sequencing. The N-terminal 10-amino acid sequences obtained agreed with those deduced from the nucleotide sequences of ORF5b and ORF6, respectively.

In Situ Activation of Diol Dehydratase-CN-Cbl Complex in E. coli Co-expressing ORF5 and ORF6 with the Diol Dehydratase Genes—E. coli JM109 carrying pUSI2E(DD) and pUSI2E (1DD), expression plasmids for the diol dehydratase genes, were co-transformed with any of the seven expression plasmids for ORF5 and/or ORF6 shown in Fig. 5A. The capability of the recombinant E. coli strains to activate the enzyme-CN-Cbl complex in situ is summarized in Table II. In the presence of free AdoCbl, ATP, and Mg<sup>2+</sup>, E. coli cells harboring plasmids containing both ORF5 and ORF6 together with the pdd genes showed a high level of activation of the diol dehydratase-CN-Cbl complex. The ability to activate the enzyme-CN-Cbl complex was not very pronounced with E. coli co-expressing ORF5 alone (pCXV(5a) and pCXV(6)) and almost negligible with E. coli co-expressing ORF6 alone (pCXV(6)) or co-expressing neither (pCXV). From these results, it can be concluded that both proteins encoded by ORF5 and ORF6 are essential for the in situ activation of the enzyme-CN-Cbl complex and therefore for the in situ reactivation of the glycerol-inactivated diol dehydratase. We propose to call these proteins a “diol dehydratase-reactivating factor.” Because this factor is encoded by ORF5 and ORF6, these ORFs were designated the ddrA and ddrB genes, respectively. A higher extent of the in situ activation was observed when ORF1 was also co-expressed with the pdd genes, ORF5 and ORF6, although co-expression of ORF1 alone with the pdd genes did not confer the reactivating activity upon E. coli cells. This indicates that the ORF1 product is not essential but stimulatory for the in situ activation of the enzyme-CN-Cbl complex.

Sequence Homologies—The deduced amino acid sequences of the reactivating factor were compared with other proteins using the FASTA program (26). The amino acid sequence of ORF5b was highly homologous to that of an ORF with an unknown function that is found immediately downstream of the glycerol dehydratase genes in the dha regulon of K. pneumoniae (Ref. 27; dhaB4, GenBank™ accession number U30903) and Citrobacter freundii (orfZ, GenBank™ accession number U09771) (identities are 61 and 59%, and similarities including the substitutions among chemically similar amino acids (28) are 78 and 77%, respectively) (Fig. 6A). The fact that these ORFs correspond to ORF5b rather than ORF5a also supports the above conclusion that the real initiation codon of ORF5 is ATG at positions 1–3 of ORF5b. An ORF6-related ORF was not found downstream of the glycerol dehydratase genes. As shown in Fig. 6B, however, ORF6 showed substantial homology to another ORF with an unknown function in the dha regulon of K. pneumoniae (orf2b, GenBank™ accession number U30903) and C. freundii (orfX, GenBank™ accession number U09771) (identities are 30 and 23%, and similarities are 47 and 44%, respectively) as well as to the β subunits of diol dehydratase (19) and glycerol dehydratase (27, 29) (identities are 25 and 20–21%, and similarities are 45 and 45%, respectively). These data suggest that the products of these ORFs are implicated in reactivation of the inactivated glycerol dehydratase.

**DISCUSSION**

We have previously reported in situ reactivation of glycerol-inactivated diol dehydratase and glycerol dehydratase and in situ activation of the enzyme-CN-Cbl complex in permeabilized K. oxytoca and K. pneumoniae cells (13, 19). Although some factors required for the in situ reactivation were suggested to be subject to induction by glycerol, isolation of the factor was impossible because the reactivating activity was not detected in vitro. In this study, we identified ORF5 and ORF6 as the genes (ddrA and ddrB genes) essential for the in situ reactivation of glycerol-inactivated diol dehydratase. Co-expression of both genes with the pdd genes conferred the diol dehydratase-reactivating activity on E. coli cells. The M<sub>r</sub> 64,000 and 14,000 polypeptides in homogenates of the recombinant E. coli cells

**Table II**

| Host/plasmids | With ATP/MgCl<sub>2</sub> | Without ATP/MgCl<sub>2</sub> |
|---------------|--------------------------|-----------------------------|
| JM109/pUSI2E(DD)/pCXV(5a) | 58 | 0.0 |
| JM109/pUSI2E(DD)/pCXV(5b) | 47 | 0.0 |
| JM109/pUSI2E(DD)/pCXV(5b-6) | 57 | 1.4 |
| JM109/pUSI2E(DD)/pCXV(5a) | 7.6 | 0.2 |
| JM109/pUSI2E(DD)/pCXV(5b) | 7.2 | 0.2 |
| JM109/pUSI2E(DD)/pCXV(6) | 0.6 | 0.2 |
| JM109/pUSI2E(DD)/pCXV | 0.5 | 0.0 |
| JM109/pUSI2E(DD)/pCXV(5a) | 86 | 0.4 |
| JM109/pUSI2E(DD)/pCXV(5b) | 89 | 0.0 |
| JM109/pUSI2E(DD)/pCXV(5b-6) | 66 | 0.2 |
| JM109/pUSI2E(DD)/pCXV(6) | 74 | 0.2 |
| JM109/pUSI2E(DD)/pCXV(5a) | 10 | 0.0 |
| JM109/pUSI2E(DD)/pCXV(5b) | 7.1 | 0.0 |
| JM109/pUSI2E(DD)/pCXV(6) | 0.0 | 0.0 |
| JM109/pUSI2E(DD)/pCXV | 1.0 | 0.0 |

*Calculated as described in the footnote to Table I.*
were characterized as the products of the \textit{ddrA} and \textit{ddrB} genes, respectively. Preliminary analysis of the homogenates by two-dimensional PAGE indicated that two polypeptides comigrated in the native dimension (nondenaturing PAGE) (data not shown), suggesting that they form a complex \textit{in vivo}. Thus, it is evident that the \textit{ddrA} and \textit{ddrB} proteins constitute the putative diol dehydratase-reactivating factor. The co-expression of ORF1 was stimulatory but not obligatory for conferring the reactivating activity on \textit{E. coli}. Thus, it was concluded that the ORF1 product is not an essential component of the reactivating factor. The function of this polypeptide remains unclear at present.

There are two possible initiation codons in ORF5: one is GTG at positions 2165 to 2163 and the other is ATG at positions 1 to 3 (Fig. 4). ORF5a and ORF5b encode polypeptides with molecular weights of 70,517 and 64,266, respectively. The polypeptides with expected sizes were detected in homogenates of \textit{E. coli} carrying pCXV(5a) and pCXV(5b), respectively. The Mr 64,000 polypeptide was predominant in \textit{E. coli} carrying pUSI2E(DD5) and also produced in \textit{E. coli} carrying pCXV(5a). These observations suggest that the ATG codon at positions 1–3 is used as the real initiation codon of ORF5. Because the \textit{pdc} and the \textit{ddr} genes are separated by 205 bp including the two inverted repeats that may form hairpin structures, the \textit{ddr} genes and the \textit{pdc} genes may be under separate control.

Such reactivating factors reported in this paper may be present in other organisms, because some of the other AdoCbl-dependent enzymes also undergo similar suicide inactivation during catalysis. One of the supporting data for this idea has recently been reported by Roth and co-workers (30). They demonstrated by a genetic study on \textit{Salmonella} that many of \textit{pduG} mutants defective in 1,2-propanediol degradation with added cyanocobalamin are corrected by exogenously supplied AdoCbl. It seems likely that the \textit{pduG} protein is related to the diol dehydratase-reactivating factor in \textit{Salmonella}. Homology searches revealed that polypeptides homologous to the \textit{ddrA} and \textit{ddrB} proteins are encoded by two ORFs with unknown functions in the \textit{dha} regulon of \textit{K. pneumoniae} (Kpn) and \textit{C. freundii} (Cfr).

Fig. 6. Comparison of the amino acid sequences of the 
\textit{ddrA} (A) and \textit{ddrB} (B) proteins with those of the \textit{\beta} subunits of diol dehydratase (DD) and glycerol dehydratase (GD) and polypeptides encoded by ORFs with unknown functions in the \textit{dha} regulon of \textit{K. pneumoniae} (Kpn) and \textit{C. freundii} (Cfr). Identical amino acids in all of the (upper) three polypeptides (A and B) and all of the six polypeptides (B) are indicated by asterisks at the top and the bottom, respectively, and similar amino acids were indicated by dots. Gaps are indicated by hyphens.
Dratase, also undergoes suicidal inactivation by glycerol during catalysis (15, 16). We have previously reported the in situ reactivation of glycerol-inactivated glycerol dehydratase in K. pneumoniae in the presence of free AdoCbl, ATP, and Mg^{2+} (13). Therefore, it seems quite reasonable to assume that the proteins homologous to the ddr proteins serve as a reactivating factor for inactivated glycerol dehydratase.

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