The N-Terminal Regions of \( \beta \) and \( \gamma \) Subunits Lower the Solubility of Adenosylcobalamin-Dependent Diol Dehydratase

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Received June 14, 2004; Accepted December 27, 2004

Adenosylcobalamin-dependent diol dehydratase is one of essential components of carboxysome-like polyhedral bodies. It exists as a heterohexamer (\( \alpha \beta \gamma \)), and its activity is recovered in a precipitant fraction of Klebsiella oxytoca and overexpressing Escherichia coli cells. Limited proteolysis of the enzyme with trypsin converted the enzyme into a highly soluble form without loss of enzyme activity. The N-terminal amino acid sequencing of the enzyme thus solubilized indicated that the N-terminal 20 and 16 amino acid residues had been removed from both the \( \beta \) and \( \gamma \) subunits, respectively. Mutant enzymes with the same N-terminal truncations of either or both of the \( \beta \) and \( \gamma \) subunits were expressed on a high level in E. coli cells. All the mutant enzymes obtained were expressed in a soluble, active form. These results indicate that the N-terminal regions of both the \( \beta \) and \( \gamma \) subunits lower the solubility of diol dehydratase. The mutant enzyme with the N-terminal truncations of both \( \beta \) and \( \gamma \) subunits was essentially indistinguishable in catalytic properties from recombinant wild-type enzyme or the enzyme purified from K. oxytoca in a soluble form.

Key words: diol dehydratase; glycerol dehydratase; solubility of enzyme; adenosylcobalamin; coenzyme B_{12}

Diol dehydratase (\( 1,2\)-propanediol hydro-lyase, EC 4.2.1.28) is an enzyme that catalyzes the adenosyl-

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate
Propionaldehyde dehydrogenase (PduP)\textsuperscript{21,22} was recombinant diol dehydratase expressed in \textit{E. coli} was also distributed mainly in the precipitate fraction and highly purified by solubilization with Brij 35.\textsuperscript{13} The X-ray structure of the enzyme revealed that substrate 1,2-propanediol and essential K\textsuperscript{+} ion are bound inside the TIM barrel in the a subunit.\textsuperscript{16} The N-terminal 45 and 36 amino acid residues of the \( \beta \) and \( \gamma \) subunits, respectively, were missing during crystallization. These regions appear not to be essential for catalytic function, because the specific activities reported with different enzyme preparations are not very much different.

In contrast, glycerol dehydratase, an isofunctional enzyme, is highly soluble.\textsuperscript{14,15,23} A comparison of the amino acid sequences of the corresponding subunits between diol and glycerol dehydratases\textsuperscript{20,23–27} showed high identities between them, except that the N-terminal 32 and 37 amino acid residues of the \( \beta \) and \( \gamma \) subunits, respectively, of diol dehydratase are lacking in the corresponding subunits of glycerol dehydratase. Hence it was suggested that these regions might determine the solubility of these enzymes, although the hydrophathy plots of diol dehydratase do not show any prominent hydrophobic peaks in these missing regions.

In this paper, we report evidence that these regions of diol dehydratase actually lower the solubility of the enzyme. By protein engineering techniques, we obtained highly soluble, truncated diol dehydratase that was expressed in \textit{E. coli}.

**Materials and Methods**

\textit{Materials.} Crystalline adenosylcobalamin (coenzyme B\textsubscript{12}) was a gift from Eisai, Tokyo. Trypsin was purchased from Sigma, St. Louis, MO. Other chemicals were analytical grade reagents. The crude membrane fraction of \textit{E. coli} carrying recombinant diol dehydratase was obtained as described previously.\textsuperscript{13}

\textit{Trypsin treatment of recombinant diol dehydratase.} Recombinant \textit{E. coli} cells expressing recombinant diol dehydratase genes were disrupted by sonication in 50 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol. The precipitate was washed three times with the same buffer. The crude membrane fraction thus obtained was re-suspended at a concentration of 5 mg of protein/ml in the same buffer containing various amounts of trypsin. After incubation at 15°C for 12 h, the reaction was terminated by the addition of excess soybean trypsin inhibitor (400 µg/ml). The resulting suspension was centrifuged at 16,000 \( \times \) g for 30 min to separate soluble and precipitant fractions.

\textit{Construction of expression plasmids.} pUSI2E(DD)\textsuperscript{20} was inserted into the \textit{BamHI}–\textit{BglII} region of pUSI2ENd to produce pUSI2ENd(DD). DNA segments encoding truncated \( \beta \) and \( \gamma \) subunits of diol dehydratase with deletions of N-terminal 20 and 16 amino acid residues, respectively, were amplified by PCR using \textit{Tag} DNA polymerase (Invitrogen, Carlsbad, CA) and the following pairs of primers: 5’-GGGACGGATATGCGACGATAAACCG-3’ and 5’-TCAGATCTTTAAAGCAGCAGCCGAC-3’ for the truncated \( \beta \) subunit gene, and 5’-TTGAGCCCATATGGAACGCCCTGC-3’ and 5’-AGAGATCTTAAATCGTCGCTTTTGAG-3’ for the truncated \( \gamma \) subunit gene (the \textit{NdeI} and \textit{BglII} sites are underlined). The 640-bp and 500-bp DNA fragments amplified were cloned into pCR2.1 vector using a TA cloning kit (Invitrogen, CA), digested with \textit{NdeI} and \textit{BglII}, and ligated with the 5.0-kb \textit{NdeI}–\textit{BglII} fragment from pUSI2ENd(DD) to produce plasmids pUSI2ENd\( (\beta’\beta’)\) and pUSI2ENd\( (\gamma’\gamma’)\), respectively. Plasmid pUSI2E\( (\alpha_0\beta’)\)\textsuperscript{13} was digested with \textit{BglII} and ligated with the 0.7-kb \textit{BamHI}–\textit{BglII} fragment obtained from pUSI2ENd\( (\beta’\gamma’)\) to yield plasmid pUSI2E\( (\alpha_0\beta’\gamma’)\). Plasmid pUSI2E\( (\alpha_0\beta’\gamma’)\) was digested with \textit{BglII} and ligated with the 0.5-kb \textit{BamHI}–\textit{BglII} fragment obtained from pUSI2ENd\( (\gamma’\gamma’)\) and pUSI2E\( (\alpha_0\beta’\gamma’)\)\textsuperscript{13} to yield plasmids pUSI2E\( (\alpha_0\beta’\gamma’\gamma’)\)\textsuperscript{13} and pUSI2E\( (\alpha_0\beta’\gamma’\gamma’)\)\textsuperscript{13}, respectively. Plasmid pUSI2E\( (\alpha_0\beta’\gamma’)\)\textsuperscript{13} was digested with \textit{BglII} and ligated with the 0.5-kb \textit{BamHI}–\textit{BglII} fragment obtained from pUSI2ENd\( (\gamma’\gamma’)\) to yield plasmid pUSI2E\( (\alpha_0\beta’\gamma’\gamma’)\). It was confirmed by sequencing that no undesired mutations occurred during the PCR amplifications.

Transformation of \textit{E. coli} with an expression plasmid and cultivation of the transformants were carried out as described previously.\textsuperscript{20}

\textit{Enzyme and protein assays.} Diol dehydratase activity was measured by the 3-methyl-2-benzothiazolinone hydrazone method using 1,2-propanediol as substrate.\textsuperscript{28} One unit of diol dehydratase is defined as the amount of enzyme activity that catalyzes the formation of 1 µmol of propionaldehyde/min. Since the solubility of wild-type diol dehydratase is very low,\textsuperscript{12,13} the homogenates, extracts, and enzyme solution to be assayed were diluted with 50 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol and 0.5–1% Brij 35. Protein was assayed by the method of Lowry et al.\textsuperscript{29} with crystalline bovine serum albumin as a standard. Specific activity was expressed as units/mg of protein.

\textit{PAGE.} PAGE of cell-free extracts was performed under non-denaturing conditions as described by Davis\textsuperscript{30} in the presence of 0.1 M 1,2-propanediol,\textsuperscript{20} or under denaturing conditions as described by Laemmli.\textsuperscript{31} Protein staining was carried out with Coomassie Brilliant Blue R-250. Western blot analysis was performed as described previously.\textsuperscript{20}

\textit{Purification of mutant diol dehydratase with the N-}
terminal truncations of $\alpha$ and $\gamma$ subunits. The cell-free extract was prepared from 3.3 g of *E. coli* cells carrying pUSI2E ($\alpha_D\beta_D\gamma_D$) by sonication. The precipitate obtained by ammonium sulfate fractionation (40–50%) of the cell-free extract was dissolved in Buffer A containing 2% 1,2-propanediol and loaded onto a Sepharose CL 6B column (bed volume, 350 ml). Fractions containing the enzyme with specific activity of more than 4 units/mg were collected. The enzyme solution was concentrated with a Centricon (Millipore, Billerica, MA) and mixed with 4 volumes of 2% 1,2-propanediol. The solution was loaded onto a DEAE cellulose (Serva) column (bed volume, 1 ml) previously equilibrated with 10 mM KPB (pH 8) containing 2% 1,2-propanediol. The column was washed with 5 volumes of the same buffer containing 50 mM KCl and then 100 mM KCl. Purified enzyme with specific activity more than 35 units/mg was used to characterize the enzyme.

**Results and Discussion**

*Limited proteolysis of diol dehydratase with trypsin*

The effect of limited proteolysis with trypsin on the solubility of diol dehydratase was investigated by incubating the suspension of a precipitant fraction of *E. coli* overexpressing diol dehydratase at 15°C for 12 h with various concentrations of trypsin. After centrifugal separation of supernatant and precipitant fractions, the diol dehydratase activity in each fraction was measured (Fig. 1A). Almost all the activity was recovered in a soluble fraction when digested with 1.0 μg/ml of trypsin.
without loss of enzyme activity. This indicates that diol dehydratase retains almost full activity after solubilization by partial tryptic digestion. Higher concentrations of trypsin, however, caused a slight loss (10–20%) of enzyme activity.

As judged from the SDS–PAGE analysis (Fig. 1B), the \( M_1 \) subunit with \( M_r \) of 30,000 and the \( \gamma \) subunit with \( M_r \) of 19,000 decreased, and instead \( M_1 \) 27,000 and \( M_1 \) 17,000 polypeptides appeared. The \( M_r \) 27,000 polypeptide appeared to be then digested to the \( M_r \) 23,000 polypeptide with 1.0–100 \( \mu \)g/ml of trypsin, and further to the \( M_r \) 21,000 polypeptide with \( \geq 200 \mu \)g/ml of trypsin. To confirm the digestion pattern, the digested subunit polypeptides were separated by SDS–PAGE, transferred to a PVDF membrane, and subjected to Edman sequencing. The N-terminal amino acid sequences of the \( M_r \) 27,000 and 23,000 polypeptides were determined to be QIIED and GSDKP, respectively. Based on the amino acid sequence of the \( \beta \) subunit, these polypeptides were identified as the \( \beta \) subunits that lack the N-terminal 9 and 20 amino acid residues, respectively. On the other hand, the \( M_r \) 17,000 polypeptide was then digested to fragments with \( M_r \)s less than 15,000. Similarly, the N-terminal amino acid sequences of the \( M_r \) 17,000 and 15,000 polypeptides determined were MNSLQG and SARVS, indicating that they are the \( \gamma \) subunits that lack the N-terminal 16 and 36 amino acid residues, respectively. The reductions in the \( M_r \)s of these \( \gamma \) subunit-derived polypeptides corresponded to the calculated molecular weights of the N-terminal polypeptides removed. But the reductions in the \( M_r \)s of the \( \beta \) subunit-derived polypeptides were larger than expected. One possibility is that the enzyme underwent partial digestion at the C-terminal region as well. Another possibility is that this discrepancy might be due to the abnormal shape of the N-terminal region of the \( \beta \) subunit. Such an abnormality was observed with the \( \beta \) subunit itself—that is, \( M_r \) of 30,000 for the \( \beta \) subunit is significantly larger than its molecular weight (24,113) calculated from the deduced amino acid sequence. In contrast to the \( \beta \) and \( \gamma \) subunits, the apparent size of the \( \alpha \) subunit appeared not to be changed, irrespective of the amount of trypsin used. The N-terminal amino acid sequence of the \( \alpha \) subunit of the solubilized enzyme was MRSKR, which agreed with the deduced N-terminal amino acid sequence. These results indicate that the \( \alpha \) subunit did not undergo digestion upon treatment with trypsin under the conditions.

In summary, the enzyme was completely solubilized without loss of activity by treatment at 15 °C for 12 h with 1.0 \( \mu \)g/ml of trypsin. The major subunit species of the solubilized enzyme were \( M_r \) 60,000 (the \( \alpha \) subunit itself), \( M_r \) 23,000 (from the \( \beta \) subunit), and \( M_r \) 17,000 (from the \( \gamma \) subunit) polypeptides. Figure 1C summarizes their fragmentation patterns in the N-terminal regions upon limited proteolysis with trypsin. These results explain why the enzyme was obtained in a soluble form with subunit heterogeneity in the former purifications, as well as the sizes of the subunits reported earlier.

Expression of mutant diol dehydratases with the N-terminal truncations of the \( \beta \) and/or \( \gamma \) subunits

The above-mentioned tryptic digestion experiment suggests that the N-terminal region(s) of the \( \beta \) and \( \gamma \) subunits lower the solubility of diol dehydratase. To examine whether the removal of the N-terminal 20 amino acid residues from the \( \beta \) subunit and/or the N-terminal 16 residues from the \( \gamma \) subunit is sufficient to make the enzyme highly soluble, we constructed expression plasmids for the mutant diol dehydratases with the N-terminal truncations of the \( \beta \) and/or \( \gamma \) subunits. pUS12E(\( \alpha_0\beta_0\gamma_0\)) and pUS12E(\( \alpha_0\beta_0\gamma_0\)) were for the expression of mutant enzymes that lack the N-terminal 20 residues of the \( \beta \) subunit and the N-terminal 16 residues of the \( \gamma \) subunit, respectively. pUS12E(\( \alpha_0\beta_0\gamma_0\)) was constructed for the expression of a mutant enzyme in which both the \( \beta \) and \( \gamma \) subunits have these N-terminal truncations.

The expression and distribution of the mutant subunits were analyzed by SDS–PAGE (Fig. 2). The density of the \( \alpha \) subunit band of all the mutant strains was almost comparable to that of the wild-type strain. As reported before, each subunit mutually affects the folding of the others in diol dehydratase. Since the \( \alpha \) subunit is the product of the gene that is located just downstream of the tac promoter, an excessive amount of the \( \alpha \) subunit is not folded correctly and thus accumulates as inclusion bodies in the precipitant fraction. The bands of truncated subunits \( \beta' \) and \( \gamma' \) in the mutant strains were not as thick as the corresponding \( \beta \) and \( \gamma \) bands in the wild-type strain. In the supernatant fractions, however, the bands of \( \beta' \) and \( \gamma' \) in the mutant strains were thicker than the

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**Fig. 2. Expression and Distribution of Mutant Diol Dehydratases with the N-Terminal Truncations of \( \beta \) and/or \( \gamma \) Subunits in E. coli.**

Proteins in cell homogenates (homo) and supernatant (sup) and precipitant (ppt) fractions of recombinant E. coli cells expressing each mutant enzyme were subjected to SDS–PAGE on an 11% gel, followed by protein staining. Experimental details are described in the text. Molecular weight markers, SDS-7 (Sigma). Positions of the \( \alpha \), \( \beta \), \( \beta' \), \( \gamma \), and \( \gamma' \) subunits are indicated with arrowheads on the right.
untruncated subunit bands of the wild-type strain. Western blot analysis using anti-diol dehydratase anti-serum showed that the Mr 23,000 and Mr 17,000 polypeptides in the supernatant fractions of mutant extracts were actually the truncated subunits C120 and C130 of diol dehydratase. Their sizes coincided well with those of the Mr 23,000 and Mr 17,000 polypeptides, respectively, formed by tryptic digestion of the enzyme. Excessive amounts of the C11 and C120 polypeptides expressed were found in the precipitant fraction, but the C130 polypeptide was not. Smaller-sized bands that reacted with anti-diol dehydratase aniserum were also observed in the supernatant fractions upon Western blot analysis.

To compare the C11/C120/C130 mutant enzyme with the wild-type (C11/C12/C13) diol dehydratase and the enzyme treated with 1.0 mg/ml of trypsin at 15°C for 12 h, cell-free extract of E. coli cells carrying pUSI2E(C11D/C120D/C130D) was subjected to PAGE under non-denaturing conditions, followed by protein staining and activity staining (Fig. 3). As shown in Fig. 3A and B, only one band showing diol dehydratase activity was observed with the mutant extract. This band co-migrated with the trypsin-treated enzyme and migrated slightly faster than the wild-type enzyme and slower than glycerol dehydratase. When the active band of mutant extract was cut out from the gel and subjected to SDS–PAGE analysis, all three polypeptides co-migrated with the Mr 60,000, 23,000, and 17,000 polypeptides of the trypsin-treated enzyme (Fig. 3C). The N-terminal amino acid sequences of the Mr 23,000 and 17,000 polypeptides in the mutant enzyme were determined to be GSDKP and MNSLQG, respectively. These coincided with the predicted N-terminal amino acid sequences of the C120 and C130 subunits, except that the N-terminal Met residue in the C120 subunit had been removed, as well as with those of the trypsin-digested enzyme. It was thus confirmed that the engineered diol dehydratase expressed in E. coli cells is catalytically active and consists of the Mr 60,000 (α), 23,000 (β: N-terminal truncated β), and 17,000 (γ: N-terminal truncated γ) subunits.

Distribution and purification of diol dehydratases with the N-terminal truncations of β and/or γ subunits
When E. coli BL21(DE3) was used as a host strain, three-fold higher activity was obtained than that with E. coli JM109 (data not shown). Hence, the former strain was used for the high level expression of mutant enzymes. E. coli BL21(DE3) carrying each plasmid was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, and analyzed for the distribution of enzyme activity after sonic disruption of the cells. As shown in Table 1, the percentages of diol dehydratase activity recovered in the soluble fraction were more than 96% with either mutant enzyme, whereas the percentage was only 2% with the wild-type enzyme. Thus it is evident that diol dehydratase becomes highly soluble when either of the N-terminal 20 residues of the C12 subunit or 16 residues of the C13 subunit are removed. This is consistent with the result of limited proteolysis with trypsin. Thus it can be concluded that both of the N-terminal regions of the β and γ subunits are necessary for precipitation or aggregation of this enzyme. It should also be noted that the total activity of mutant enzymes was lower than that of the wild-type enzyme. Since the enzyme with N-terminal truncations of the β and γ subunits was almost fully active (Fig. 1A), perhaps the mutant enzymes expressed in soluble forms are more susceptible to proteolysis than the wild-type enzyme.
This speculation is consistent with the fact that threefold higher activity was obtained when the wild-type enzyme was expressed with *E. coli* BL21(DE3) as a host, because this strain lacks lon and ompT proteases.\(^{33,34}\)

Partially purified αβ′γ enzyme was obtained from the cell-free extract of *E. coli* cells carrying pUSI2E(αβDβD′D′) by ammonium sulfate fractionation, Sepharose CL-6B column chromatography, and DEAE-cellulose column chromatography. The specific activity of this preparation of αβ′γ was 47 units/mg. Densitometric analysis after PAGE showed that the purity of the αβ′γ band was 55%. These results suggest that the specific activity of the homogeneous preparation of αβ′γ enzyme is 85 units/mg, which is comparable to that of the enzyme purified from *K. oxytoca* in a soluble form (91.5 units/mg)\(^{18}\), and of the purified recombinant wild-type enzyme (106 units/mg)\(^{13}\). The \(K_m\) values for a substrate (DL-1,2-propanediol) obtained with αβ′γ and wild-type enzymes were 0.11 and 0.10 mM, respectively, both of which values are reasonably consistent with the value obtained with the purified soluble enzyme from *K. oxytoca* (0.18 mM)\(^{2}\). From the data described above, it was concluded that the mutant enzyme with the N-terminal truncations of both β and γ subunits is essentially indistinguishable in catalytic properties from recombinant wild-type enzyme or the enzyme purified from *K. oxytoca* in a soluble form. However, the \(K_m\) value for AdoCbl obtained with the αβ′γ enzyme was 0.28 μM, smaller than that obtained with wild-type enzyme (0.83 μM).\(^{13}\) The \(K_m\) value for the coenzyme with the enzyme purified from *K. oxytoca* in a soluble form is 0.80 μM.\(^{20}\) The reason for this difference is not clear.

At present, it is not clear why both of the N-terminal regions of the β and γ subunits are required for making this enzyme almost insoluble. This is an enigma because no prominent hydrophobic peaks were found in these N-terminal regions of either subunits as judged from their hydrophathy profiles. Hence this point appears crucial for our understanding of the way of protein assemblies around diol dehydratase in the polyhedral bodies in bacterial cells. We have reported the X-ray structures of diol dehydratase in complexes with cyanocobalamin\(^{16}\) and adeninylpentylcobalamin.\(^{35}\) The structures of the N-terminal regions of the β and γ subunits remain unclear, because this enzyme becomes crystallized only after cleavage of the N-terminal 45 residues of the β subunit and the 36 residues of the γ subunit.\(^{16}\) These regions are considered to extend to the solvent and are believed to be inhibitory for compact filling up in crystallization.\(^{16}\) When the Cα atoms of the N-terminal amino acids (Gβ46 and Syγ37) of the β and γ subunits located on an electron density map are indicated by grey and black balls, respectively, in the X-ray structure of diol dehydratase (Fig. 4A), it is evident that the N-terminal regions of both subunits are located roughly at the tops of tetrahedral structure. That is, the two tops are occupied by the N-terminals of the two β subunits and the other two by those of the two γ subunits (Fig. 4B). Such steric positions might be important for the formation of large protein assemblies by intermolecular interactions within the enzymes and/or of the enzyme with other protein components in the polyhedral bodies.

**Acknowledgments**

This work was supported in part by Grants-in-Aid for Scientific Research ((B) No. 13480195 and (C) No. 14580627, and Priority Areas No. 753) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Professor Hidenori Yamada for N-terminal sequencing and Ms. Yukiko Kurimoto for her assistance in manuscript preparation.
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