Stability-increasing Mutants of Glucose Dehydrogenase from Bacillus megaterium IWG3*

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A glucose dehydrogenase gene was isolated from Bacillus megaterium IWG3, and its nucleotide sequence was identified. The amino acid sequence of the enzyme deduced from the nucleotide sequence is very similar to the amino acid sequence of the enzyme from Bacillus megaterium M1286 reported by Jany et al. (Jany, K.-D., Ulmer, W., Froschle, M., and Pfeiderer, G. (1984) FEBS Lett. 165, 6–10). The isolated gene was mutagenized with hydrazine, formic acid, or sodium nitrite, and 12 clones (H35, H39, F18, F20, F191, F192, N1, N13, N14, N28, N71, and N72) containing mutant genes for thermostable glucose dehydrogenase were obtained. The nucleotide sequences of the 12 genes show that they include 8 kinds of mutants having the following amino acid substitutions: H35 and H39, Glu-96 to Gly; F18 and F191, Glu-96 to Ala; F20, Gln-252 to Leu; F192, Gln-252 to Gly; N1, Glu-96 to Lys and Val-183 to Ile; N13 and N14, Glu-96 to Lys, Val-112 to Ala, Glu-133 to Lys, and Tyr-217 to His; N28, Glu-96 to Lys, Asp-108 to Asn, Pro-194 to Gln, and Glu-210 to Lys; and N71 and N72, Tyr-253 to Cys. These mutant enzymes have higher stability at 60 °C than the wild-type enzyme. The results of a study indicate that the tetrameric structure of glucose dehydrogenase is stabilized by several kinds of mutation, and at least one of the following amino acid substitutions stabilizes the enzyme: Glu-96 to Gly, Glu-96 to Ala, Gln-252 to Leu, and Tyr-253 to Cys.

Enhancement of stability of enzymes is one of the most popular targets for redesigning enzymes by protein engineering (1). Many mutant enzymes with enhanced stability have been obtained by genetic screening and site-directed mutagenesis, and the mechanism of the enhancement has been explained as the increase in the free energy of stabilization of a native tertiary structure for reversible denaturation, or as the increase in the resistance to chemical and enzymatic modifications causing irreversible inactivation (1, 2). For oligomeric enzymes, however, another mechanism is possible; that is, the stabilization of their oligomeric structures by strengthening intersubunit association.

Glucose dehydrogenase (EC 1.1.1.47) from Bacillus megaterium is a tetrameric enzyme with four identical subunits (3). The enzyme is inactivated in alkaline solutions and is stabilized by the addition of NaCl (4, 5). It has been reported that the inactivation is due to the reversible dissociation of the tetramer into inactive protomers, and the mechanism of the reversible dissociation has been investigated by Pfeiderer (4, 6, 7). Therefore, this enzyme is a good candidate to be redesigned to a more stable structure using the new mechanism of strengthening intersubunit association.

In this work, we isolated a glucose dehydrogenase gene from Bacillus megaterium IWG3. As the tertiary structure of this enzyme is not known, we treated the gene with chemical mutagens, and obtained 12 transformant colonies producing mutant enzymes with enhanced stability. These mutant enzymes provided us with information on the amino acid residues that affect the stability of the enzyme by changing the tertiary structure of the protomer and/or by changing the strength of the intersubunit interaction.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Glucose dehydrogenase from Bacillus megaterium IWG3 was a generous gift from Amano Pharmaceutical Co. Ltd. (Nagoya), and was used after purification by DEAE-Sephadex A-50 column chromatography and HPLC (3) (G3000SW and DEAE-3SW). Restriction endonucleases (BanI, BglII, EcoRI, HaeIII, HpaII, HindIII, PstI, PvuII, RsaI, SalI, and SacI) and T4 DNA ligase were obtained. Polynucleotide kinase, the Klenow fragment, mung bean nuclease, and P1 primer were purchased from Toyobo Co. Ltd. (Osaka); reactions of the restriction endonucleases and ligase were done as described in Ref. 8. NADP was purchased from Oriental Yeast Co. Ltd. (Tokyo). NAD was a generous gift from Kojin Co. Ltd. (Tokyo). Other chemicals were obtained from Nakarai Chemical Co. Ltd. (Kyoto).

Bacterial Strains, Phages, and Plasmids—B. megaterium IWG3 was used as the source of a glucose dehydrogenase gene. The following bacterial strains, phages, and plasmids were used: Escherichia coli strains C600ompZ (thr-1 leuB6 thi-1 supE44 lacY1 tonA21 hsdR16), JM103 (Δlac-pro thi strA supE endA sbcB15 hsdR4, F' traD36 proAB lacI121 lacZ165) (10), and JM105 (Δlac-pro thi strA sbcB15 hsdR4, F' traD36 proAB lacI121 lacZ165) (11); M13mp18 and mp19 phages; and pBR322 (12) and pKX223-3 (13).

Preparation of Plasmid and Phage DNAs—Plasmid DNAs were prepared by the alkaline-extraction method described by Birnboim and Doly (14) and further purified by CsCl/ethidium bromide density gradient centrifugation (15). M13 phage DNAs were prepared as described in Ref. 16.

Preparation of Probe DNA—The amino acid sequence of the N-terminal region of glucose dehydrogenase was identified by sequential Edman degradation using an automated protein sequencer (Applied Biosystem, model 470A); the sequence (1–29) was found to be the same as that reported by Jany et al. (17). The probe for the N-terminal region of the enzyme (5'-GTTAATAACAACAACTTTCTTTCTCCATCTTTATAACAT-3') (38-mer) was chemically synthesized with an automated DNA synthesizer (Applied Biosystem, model 381A), and purified by reverse-phase HPLC (Cosmosil 5C4-300). The purified oligonucleotide was labeled using γ-32P-ATP (5000 Ci/mmol) and T4 polynucleotide kinase (18).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04805.

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‡ The abbreviations used are: HPLC, high-performance liquid chromatography; kb, kilobase pair.
mmol, ICN) and polynucleotide kinase.

Transformation—E. coli strains were transformed by the CaCl₂ method of Cohen et al. (18). Transformants harboring hybrid plasmids of pBR322 were selected on LB agar medium (19) containing 50 μg/ml of ampicillin. M13 phage plaques were selected on an agar medium containing 1% Bactotryptone, 0.8% NaCl, 0.1% maltose, 1% 3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mM 5-ethylphenazinium ethylsulfate, and 0.2 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described in Ref. 16.

Filter Assay of Glucose Dehydrogenase Activity—Glucose dehydrogenase activity of transformants were detected as described in Refs. 20 and 21. Colonies on a plate were transferred to a paper filter, and the cells were lysed at room temperature. Then the filter was incubated at 60 °C for 20 min in 50 mM potassium phosphate, pH 6.5, containing 2 mM NaCl and 50 mM EDTA (heat treatment). Positive clones were selected by incubating the filter at 30 °C for 10 min in 20 mM Tris/HCl, pH 8.0, 1 M NaCl, 0.1 M D-glucose, 0.5 mM 3-[(4',5' dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium bromide, 0.5 mM 5-ethylphenazinium ethylsulfate, and 50 μM NAD.

Isolation of a Glucose Dehydrogenase Gene—Chromosomal DNA of B. megaterium IWG3 prepared by the method of Saiito and Miura (22) was digested with EcoRI and BglII. After agarose gel (1%) electrophoresis (8) of the digested DNA, 3-4 kb fragments that hybridized with the synthetic DNA probe were obtained by extraction from the gel by the method of Vogelstein and Gillespie (23) using a Geneclean kit (Bio101 Inc.). The DNA fragments were ligated with pBR322 that had been digested with EcoRI and BamHI, and E. coli C600 was transformed with the ligated DNA. Ampicillin-resistant transformants (about 350 colonies) were analyzed by colony hybridization (24) with the probe DNA at 45 °C, and three positive colonies were obtained. These three clones were further tested for glucose dehydrogenase activity by the filter assay, and two of them were found to be positive. Since the plasmids from the two clones showed the same restriction pattern for BamHI, HindIII, PstI, and PvuII digestion, we selected one of the clones and named the plasmid pGDA1. Southern hybridization analysis (25) showed that the 3.6-kb EcoRI-SalI fragment of pGDA1 strongly hybridizes with the probe DNA.

Subcloning of the Glucose Dehydrogenase Gene—pGDA1 was digested with EcoRI and PstI. A 1.5-kb DNA fragment was obtained by agarose gel electrophoresis as described above. The terminal single-stranded regions of the fragment were filled in by the Klenow fragment. The DNA fragment was then ligated with the PstI linker, and digested with BamHI. The resulting terminal single-stranded regions of the fragment were removed by manganese nuclelease, the fragment was ligated with the EcoRI linker and digested with EcoRI and PstI. The EcoRI-PstI fragment with 0.9 kb was inserted into pKK223-3, and E. coli JM105 was transformed with the hybrid plasmid. The glucose dehydrogenase activity of the transformants grown on an LB (18) plate containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactoside was detected by the filter assay. A plasmid harbored in one of the positive clones were selected and named pGDA2.

Chemical Mutagenesis—The EcoRI-PstI 0.9-kb DNA fragment containing the glucose dehydrogenase gene was inserted into M13mp18 and M13mp19. The single-stranded DNA of the hybrid plasmid was treated at 20 °C with hydrazine (for 5-20 min), formic acid (5-20 min), or sodium nitrite (1-3 h) by the method of Myers et al. (26). The mutagenized single-stranded DNA was annealed with the P1 primer and made into a duplex form as described in Ref. 26. The duplex DNA was digested with EcoRI and PstI, the resulting 0.9-kb fragment was inserted into pKK223-3, and E. coli JM105 was transformed with the hybrid plasmid.

RESULTS AND DISCUSSION

Glucose Dehydrogenase Gene from B. megaterium—We have isolated a glucose dehydrogenase gene from B. megaterium IWG3 as described under "Experimental Procedures," and cloned it in a plasmid, pGDA1. Fig. 1 shows the physical map of pGDA1. Southern hybridization experiments (25) using the synthetic DNA probe showed that the 0.5-kb BamHI-HindIII fragment encodes the N-terminal region of the enzyme. Therefore, we identified the nucleotide sequence of both strands of the 0.93-kb region flanked by EcoRI and Sau3A1 (Fig. 1) by the dideoxynucleotide chain-termination method (27) using a sequence kit (Toyobo Co. Ltd.); the sequence strategy is also shown in Fig. 1.

FIG. 1. Restriction map of pGDA1 and sequencing strategy for the glucose dehydrogenase gene. pGDA1 is a hybrid plasmid that consists of pBR322 (solid bar) and an EcoRI-BglII fragment (3.3 kb) of the chromosomal DNA from B. megaterium I WG3 (open box). The black box represents the DNA fragment that hybridizes with the synthetic DNA probe. The dotted box represents the structural gene of glucose dehydrogenase. The arrows indicate the direction and the extent of the sequencing.
Fig. 2 shows the nucleotide sequence of the 0.93-kb fragment. There is an open reading frame of 783 base pairs available to encode a peptide of 261 amino acids (Mr 28,085) which starts from ATG at position 60 and ends at TAA at position 843. This size agrees well with the Mr of the subunit of the purified glucose dehydrogenase (Mr 30,000). The 29 amino acids of the N-terminal region deduced from the nucleotide sequence are the same as those identified by Edman degradation of the purified protein (the underlined amino acid sequence shown in Fig. 2). A possible ribosome-binding sequence (AGGAGG) is identified 9 bp upstream from the initiation codon. Downstream from the coding region, there is an inverted repeat sequence (16 base pairs each) followed by an AT-rich sequence that seems to be a transcriptional termination signal.

Chromosomal DNA of B. megaterium IWG3 was digested with HindIII or EcoRI + PstI, and analyzed by Southern hybridization (25) at 60 °C using the nick-translated 0.9-kb EcoRI-PstI fragment from pGDA2 as a probe DNA (Fig. 3). The results show that there are three hybridization signals other than that for the isolated gene (0.7 kb for the HindIII digestion and 3.4 kb for the EcoRI + PstI digestion): 0.8, 1.6, and 3.5 kb for the HindIII digestion, and 1.2, 2.0, and 4.6 kb for the EcoRI + PstI digestion. These additional signals show that B. megaterium IWG3 has at least two or three additional isozyme genes of glucose dehydrogenase. Recently, Heilmann et al. (28) cloned two glucose dehydrogenase genes from B. megaterium M1286. The presence of several isozyme genes of glucose dehydrogenase may have some physiological meaning.

The amino acid sequence of glucose dehydrogenase from B. megaterium IWG3 can be deduced from the nucleotide sequence, and is similar to those from B. megaterium M1286 (17, 28, 29) and B. subtilis (50); the amino acids that are not conserved in all four sequences are marked with asterisks in Fig. 2. The values of the sequence homology are about 83% between our enzyme and glucose dehydrogenase A from B. megaterium M1286 (28) and 82% between ours and the glucose dehydrogenase from B. subtilis (30). Our enzyme is almost the same as one of the glucose dehydrogenases from B. megaterium M1286 whose protein sequence has been reported in Ref. 17; only 1 amino acid out of 261 is different: Leu-95 in our sequence is changed to Met-95 in the reported protein sequence (17) with some corrections (28, 29). In spite of these high similarities, the reported protein sequence has two insertions (Glu-52 and Trp-150) compared with our sequence. This seems to be a very rare case, and must be a special example for enzyme evolution, provided these sequences are correct.

Isolation and Characterization of Genes for Thermostable Mutants—The EcoRI-PstI 0.9-kb DNA fragment was treated with hydrazine, formic acid, or sodium nitrite as described under "Experimental Procedures." The transformants (about 20,000 colonies) harboring the hybrid plasmid containing the mutagenized glucose dehydrogenase gene were analyzed for heat-resistant enzyme activity by the filter assay as described under "Experimental Procedures" with one alteration, that 2 M NaCl was omitted from the solution for the heat treatment at 60 °C. Under these conditions, native enzyme is inactivated and cannot be detected by the filter assay. Among the transformants, we obtained 12 positive clones; two of them were obtained from the hydrazine treatment (about 4,000 transformants), four from the formic acid treatment (about 7,500 transformants), and six from the sodium nitrite treatment (about 8,000 transformants).

We identified the nucleotide sequences of both strands of the EcoRI-PstI fragments (0.9 kb) from the 12 plasmids by the method of Henikoff (31). Table I summarizes the base substitutions caused by the chemical mutagenesis. Of the 12 clones, four pairs have identical genes: H35 and H39, F18 and F191, N13 and N14, and N71 and N72. There are two possibilities for getting these duplicate clones: the same pattern of base substitution occurred on two or more DNA fragments coding the glucose dehydrogenase gene, or transformants were duplicated by growth before the plate culture. The paired clones were obtained from one chemical mutagenesis experiment, and so we cannot select one of the possibilities.

We have thus obtained 8 kinds of mutant genes coding for thermostable glucose dehydrogenases. Among 26 base substitutions, 21 are transitions and 5 are transversions. All the transitions were made by the mutagenesis with hydrazine and sodium nitrite, and all the four types of transitions were observed (Table I). On the coding strand, however, there was no C to T transition. Four transversions were made by the mutagenesis with formic acid, one was from the mutagenesis with sodium nitrite, and a total of four types out of eight were observed (Table I). We mutagenized both coding and noncoding strands of the EcoRI-PstI DNA fragment using M13mp18 and M13mp19, respectively. This also increased the variety of mutant genes.

Not all of the base substitutions observed caused amino acid substitutions, and some of the base substitutions are silent when a mutant gene has several base substitutions. Table I also shows the amino acid substitutions observed in the mutant genes coding thermostable glucose dehydrogenases. The increased thermostability of the mutant enzymes was confirmed by measuring the remaining activity after heat treatment (at 60 °C for 20 min in 50 mM sodium phosphate buffer, pH 6.5) of crude enzyme solutions. After this heat treatment, the remaining activity of wild-type enzyme cannot be detected, but those of the mutant enzymes are 1–97% (Table I). These mutant enzymes seem to be classified into at least three groups based on the thermostability.

Jany et al. (17) have reported that the modification of Tyr-254 (in our enzyme Tyr-253) with tetranitromethane completely inactivates the enzyme without affecting the overall
structure, and they concluded that Tyr-254 is involved either in catalysis or binding of substrates (17). The mutant N72, however, has the substitution of cysteine for Tyr-253 and has activity. These results indicate that Tyr-253 is not essential for the catalytic function. As the primary structures of these enzymes are almost the same, this discrepancy is not due to the difference in the structures of the two enzymes; the inactivation by the chemical modification of Tyr-254 may be due to a change in the conformation of the active site. The substitution of cysteine for Tyr-253, on the other hand, does not cause such a fatal change in the active site but increases the heat resistance of the enzyme, probably because cysteine is smaller than tyrosine. The role of Tyr-253 will be clarified by making mutant enzymes with different amino acids at this position.

Fig. 2 shows that the amino acid substitutions cluster in a central region (96–133) and the C-terminal region (183–258), and no substitutions are observed in the N-terminal region (1–95) although there are two silent base substitutions in N28. In contrast, Fig. 2 also shows that 45% of the amino acids not conserved in all the four sequences are in the N-terminal region. This means that many amino acids in the N-terminal region can be replaced by others without large losses of activity, but none of the substitutions can increase the heat resistance of the enzyme. When a mutant enzyme has two or more amino acid substitutions, some of them may be neutral mutations that do not affect the activity and stability of the enzyme very much. Therefore, some mutants having additional neutral mutations in the N-terminal region could have been obtained, but they were not. This is an interesting phenomenon but we have to prepare more mutants to understand the role of the N-terminal region in the stability of the enzyme. It is also noteworthy that the mutated amino acid positions except Asp-108 and Gln-252 are all conserved in all the four sequences (Fig. 2). These amino acids are probably conserved because the stability of the enzyme must be kept at a desired level; that is, too stable a glucose dehydrogenase may not be good for the bacteria.

Among 13 kinds of amino acid substitutions that increase the heat resistance of the enzyme, 8 substitutions decrease the number of negative charges and 4 of the 8 increase the positive charge of the enzyme at pH 6.5. These decreases in the total negative charge seem to contribute to the stabilization of the tertiary and quaternary structures of the enzyme by modulating charge-charge interactions, considering that this enzyme has an acidic isoelectric point (pI 4.7) (3) and is stabilized by a high ionic concentration (4, 5). The mutants F20 and F192, on the other hand, have the same charge as the wild-type enzyme, and therefore, the stabilization mechanism of these mutants must be different from the others.

To know the effects of each amino acid substitution in the mutants, F192, N1, N13, and N28, we must prepare the mutants each having one of the substitutions. The effects of the combination of these mutations including those in H35, F18, F20, and N71 are also interesting. The mutation at Glu-96 is observed most frequently. To remove the negative charge, position 96 must be important for the increase in the stability. It is noteworthy that the mutant F18 has a higher stability than H35. This means that the methyl group of Ala-96 contributes to the increase in the stability; this stabilization mechanism is also interesting. These mutations may affect not only the stability but also the other properties of the enzyme. These properties of the mutant enzymes will be studied after purification of these enzymes.

In this work, we found that a single mutation of Glu-96 to glycine or alanine, Gln-252 to leucine, or Tyr-253 to cysteine increases the heat resistance of glucose dehydrogenase. These results indicate that any one of the four mutations can sta-
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bilize the tetrameric structure of the enzyme. Glu-96, Gin-252, and Tyr-253 are conserved in all the four glucose dehydrogenases except that Gln-252 is changed to lysine in the sequence of glucose dehydrogenase A from B. megaterium M1286 (Fig. 2). Therefore, these enzymes will also be stabilized by the above stability-increasing mutations.

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