Gene Cloning and Characterization of the Very Large NAD-Dependent L-Glutamate Dehydrogenase from the Psychrophile Janthinobacterium lividum, Isolated from Cold Soil

Ryushi Kawakami, Haruhiko Sakuraba and Toshihisa Ohshima

Glutamate dehydrogenases (GDHs) catalyze the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonia using NAD(P) as a coenzyme (7, 11, 13, 21). The enzymatic activity of GDHs is well characterized, and GDHs have been classified into three groups based on their coenzyme specificity: NAD-dependent GDHs (EC 1.4.1.4) are involved in ammonia assimilation and are distributed in eukarya, bacteria, and archaea (7); NAD(P)-dependent GDHs (EC 1.4.1.3) are distributed in the mitochondria of mainly euukaryotic cells (22); and NAD-dependent GDHs (NAD-GDH) (EC 1.4.1.2) are involved in glutamate catabolism and are distributed in plants, animals, and bacteria (11, 13). Almost all NADP- and NAD(P)-dependent GDHs from archaea to eukarya are known to be hexamers with subunit molecular masses of about 50 kDa. On the other hand, NAD-GDHs are classified into three subgroups with different molecular masses and oligomeric structures (5). Although most known types of NAD-GDHs from a variety of organisms occur as hexamers with subunit molecular masses of about 50 kDa (GDH-50s), those from several euukaryotic microorganisms, such as Neurospora crassa and Saccharomyces cerevisiae, exhibit a tetrameric structure with subunit molecular masses of about 115 kDa (5, 8, 10, 12, 18, 25). In addition, a third group of NAD-GDHs with subunit molecular masses of about 180 kDa (GDH-180s) have been found in three bacteria: Pseudomonas aeruginosa (17), Streptomyces clavuligerus (19), and Psychrobacter sp. strain TAD1 (6). There is currently much less functional and structural information about GDH-180s than about the other two GDH groups.

Up to now we have been investigating the enzymological and structural characteristics of GDHs from hyperthermophiles such as Pyrococcus furiosus (21), Pyrobaculum islandicum (3, 14), and Aeropyrum pernix (2). These enzymes exhibit a high degree of activity at temperatures around 100°C, but the catalytic activity markedly declines as the temperature is reduced. By contrast, enzymes from psychrophiles are generally thermostable and easily lose activity as the temperature is increased, but they often exhibit a high degree of catalytic activity at temperatures well below room temperature. We are therefore interested in the catalytic properties of GDHs from psychrophiles and in the differences in the catalytic and structural characteristics of the psychrophilic and thermophilic enzymes.

We isolated a psychrophile, Janthinobacterium lividum
followed by a 60-s rest at 10°C, and any remaining intact cells and the cell debris were disrupted by sonication in 10 mM glycine/NaOH (pH 9.5), 1 mM NAD, and 2 U of NADH oxidase, after which the cells were disrupted by sonication (20 g [wet weight] in 100 ml of the standard buffer) to a final concentration of 1 mM, after which the cells were disrupted by sonication in 10 mM glycine/NaOH (pH 7.0), and NAD at 3°C during glutamate oxidation. The protein concentration was determined by the method of Bradford (4); bovine serum albumin served as the standard.

Purification of GDH. All steps in the purification procedure were carried out at 4°C. Phenylmethylsulfonyl fluoride in dimethyl sulfoxide was added to the cell suspension (20 g [wet weight] in 100 ml of the standard buffer) to a final concentration of 1 mM, after which the cells were disrupted by sonication (TOMY UD-200; Tomy, Tokyo, Japan) using five cycles of 60-s pulses (35 W) following a 60-s rest at 4°C, and any remaining intact cells and the cell debris were removed by centrifugation (20,000 × g for 20 min at 4°C). The resultant supernatant was used as the crude extract and applied to a DEAE-Toyopearl column equilibrated with the standard buffer. The column was then washed with the standard buffer, and the enzyme was eluted with a linear 0.5 to 0.1 M NaCl gradient in the same buffer. The active fractions were pooled, and solid ammonium sulfate was added to 20% saturation while the pH was maintained at 7.0 by the addition of 14% NH4OH. The enzyme solution was applied to a Toyopearl column previously equilibrated with the standard buffer supplemented with 20% ammonium sulfate. After the column was washed with the same buffer, the enzyme was eluted with a linear 20 to 0% ammonium sulfate gradient in the same buffer. The active fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol. The dialyzed protein was added to a Blue Sepharose CL-4B column equilibrated with the same buffer, after which the column was washed with the same buffer and the enzyme was eluted with the buffer containing 1.5 M NaCl. Active fractions were pooled and dialyzed against the standard buffer (pH 7.0).

Electrophoresis and determination of molecular mass. Native polyacrylamide gel electrophoresis (PAGE) was carried out at 4°C on a 7.5% polyacrylamide gel using the method of Davis (9), after which the proteins were stained with Coomassie brilliant blue R-250. Active staining was performed at 25°C using a mixture containing 300 mM Tris-HCl (pH 8.0), 50 mM L-glutamate, 0.1 mM p-iodonitrotetrazolium violet, 0.04 mM phenazine methosulfate, and 0.25 mM NAD. Sodium dodecyl sulfate (SDS)-PAGE was carried out on a 10% polyacrylamide gel using the method of Laemmli (15). Maltose-binding protein-β-galactosidase (175 kDa), maltose-binding protein-paramyosin (83 kDa), glucosamine oxidase (62 kDa), aldolase (47.5 kDa), triose phosphate isomerase (32.5 kDa), D-fructose 1,6-bisphosphatase (52 kDa), isocitrate lyase (16.5 kDa), and aprotinin (6.5 kDa) were used as the molecular standards (New England Biolabs). The molecular mass of the enzyme was determined by Superose 6 gel filtration chromatography using 10 mM potassium phosphate (pH 7.0) and 0.2 M NaCl as the elution buffer. Thryoglibulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) were used as the molecular mass standards (Amersham Biosciences); bovine α-crystallin (about 810 kDa) was also used as a high-molecular-mass standard.

Terminal amino acid sequence analysis. The N-terminal amino acid sequence of the isolated enzyme was analyzed using an automatic Edman degradation protein sequencer. The phenylthiohydantoin derivatives were separated and identified using a Shimadzu PPSQ-10 protein sequencer (Kyoto, Japan).

Cloning of the GDH gene. Genomic DNA from J. lividum UTB1302 was prepared using a GenomicPrep cell and tissue DNA isolation kit according to the manufacturer's instructions. To clone the GDH gene, an oligonucleotide mixture probe (probe 1) was labeled with 32P and used for hybridization. Several colonies containing the GDH gene were selected. Preparation of probes was performed using the method of Utb1302. The sequence of the known GDH gene fragment was cloned. These sequence data were then analyzed using GENETYX-MAC (Software Development, Tokyo, Japan). Expression of the GDH gene and product purification. The GDH gene was amplified by PCR using genomic DNA as the template with the following primers: 5'-ATGACTGATATCCATTCAAGCTTCTTA-3' (sense) and 3'-TAATGGAGATGCCATATGAATCACACGCCA-5' (reverse). The forward primer introduced a unique NdeI restriction site that overlapped the 5' initiation codon, and the reverse primer introduced a unique BamHI restriction site proximal to the 3' end of the termination codon. After PCR using Pfu Turbo DNA polymerase, the amplified DNA fragment was confirmed from its sequence, digested with NdeI and BamHI, and inserted into pCold IV (Takara) to produce the expression plasmid pCold/JLGDH. L. coli TOP10 competent cells were then transformed with pCold/JLGDH, and the transformants were grown in Luria-Bertani medium containing 0.01% ampicillin and 0.02% IPTG. One of the SphI fragments containing the GDH gene was cloned. A plasmid pCold/JLGDH was then synthesized based on the 3' sequence of the known GDH gene fragment, and the screening protocol was repeated until the entire length of the GDH gene was cloned. The sequence data were then analyzed using GENETYX-MAC (Software Development, Tokyo, Japan).
Table 1. Physiological characteristics of *J. lividum* UTB1302.

<table>
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<tr>
<th>Characteristic</th>
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<td>Nitrate reduction</td>
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<td>Indole production</td>
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Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA and GDH genes have been submitted to DDBJ under accession numbers AB286844 and AB286655, respectively.

RESULTS

Screening of psychrophilic GDH. By screening at 4°C, we were able to isolate 78 psychrophile strains from low-temperature soil collected from Tsugaika kougen in a mountainous region of Nagano Prefecture in Japan. These strains were then cultured in liquid medium at 4, 20, 25, 30, and 35°C, and the 16 strains that grew well at 4°C but did not grow at temperatures above 30°C were selected for a second screening. Crude extracts were prepared from these 16 strains, and GDH activity was detected using PAGE with active staining and with NAD or NADP as a coenzyme. NAD- or NADP-dependent GDH activity was observed in the crude extracts of seven strains. Of these, strain No. 68 was able to grow at 0°C but not at temperatures above 30°C and was chosen as a candidate producer of psychrophilic GDH. To identify strain No. 68, we determined its physiological characteristics (Table 1) and the sequence of its 16S rRNA gene (991 bp). We found that the sequence was completely identical to that of *J. lividum* and that the physiological properties were not contradictory to those of *J. lividum* as far as they are known (data not shown). Thus, strain No. 68 was identified as *J. lividum* UTB1302.

Purification of GDH from *J. lividum* UTB1302. *J. lividum* UTB1302 cells (20 g, wet weight) were collected from 2 liters of culture medium, and about 2,000 mg of soluble protein was obtained in the crude extract after sonication and centrifugation. The purified enzyme was then obtained from the extract using the four-step procedure described in Materials and Methods. The purified GDH migrated as a single band on both native PAGE and SDS-PAGE gels and was homogeneous (Fig. 1). The subunit and native molecular masses of GDH were determined to be about 170 kDa (by SDS-PAGE) and about 1,065 kDa (by Superose 6 gel filtration chromatography), respectively, which indicates that the native enzyme occurs as a homohexamer. The N-terminal amino acid sequence of the enzyme was determined to be MNHTQDLXTQXL (where X is an unidentified amino acid residue).

Cloning of the GDH gene from *J. lividum* UTB1302 genomic DNA. To clone the GDH gene, we initially synthesized a degenerate oligonucleotide probe (probe 1) based on the N-terminal amino acid sequence of the enzyme. After Southern hybridization of the probe, a 2.5-kbp SphI fragment of genomic DNA showed a positive signal. Subsequent colony hybridization yielded a positive clone (pgdh1), and once inserted into pUC18, the DNA was sequenced. The fact that the amino acid sequence deduced from the nucleotide sequence included the known N-terminal amino acid sequence confirmed that we had determined part of the amino acid sequence (694 amino acids) of *J. lividum* GDH. Thereafter, a new probe (probe 2; 5′-GA ACTCGAAGCGGCATCTCC-3′) was synthesized based on the nucleotide sequence of the known fragment, and cloning yielded pgdh2, in which a 0.5-kbp SalI fragment of genomic DNA was inserted. We then sequenced this fragment and deduced the amino acid sequence (175 amino acids). Using the same method, pgdh3 containing a 3.0-kbp SphI fragment of the genomic DNA was obtained, from which a 706-amino-acid sequence was deduced. In addition, a stop codon (TAA) was found within the nucleotide sequence.

Ultimately, we were able to clone the entire GDH gene using the three genome fragments (Fig. 2). The total length of the gene was 4,725 bp, and the deduced amino acid sequence contained 1,575 amino acids with a molecular mass of 169,360 Da, which corresponded well to the molecular mass determined by SDS-PAGE (170 kDa). A BLAST search revealed that the amino acid sequence of *J. lividum* GDH showed homology with the sequences of known NAD-dependent GDHs from *S. clavuligerus* (38%) and *P. aeruginosa* (38%) and with the sequences of putative GDHs from *Chromobacterium violaceum* ATCC 12472 (40%), *Ralstonia metallidurans* CH34 (41%), *Shewanella putrefaciens* CN-32 (40%), *Burkholderia cenocepacia* HI2424 (41%), *Xanthomonas campestris* pv.
FIG. 2. Nucleotide and deduced amino acid sequences of *J. lividum* GDH. The N-terminal amino acid sequence determined by protein sequencing is underlined. The full nucleotide sequence was obtained by sequencing pgdh1, pgdh2, and pgdh3, which were comprised of genomic SphI (nucleotide positions 1 to 2295), SalI (nucleotide positions 2243 to 2821), and SphI (nucleotide positions 2296 to 5098) fragments, respectively. The amino acid sequence of the central domain (amino acids 719 to 1190) is shaded.
amides (10 mM) were examined in the presence of NAD: L-

of 16 mg of purified protein was obtained from 3 g (wet weight)

The recombinant GDH was easily purified by successive

TOP10 cells transformed with empty pCold IV (negative con-

dependent GDH activity was detected in the crude extract of

cells (Table 2 and Fig. 3).

The activity of the purified recombinant GDH was maximal

at 40°C in a medium containing 5 mM NAD. The

Effects of various other compounds on the enzyme ac-

activity also were examined (Table 3). Activation of GDH activity

was observed when L-aspartate, L-arginine, L-tryptophan, L-

methionine, L-lysine, L-histidine, or D-arginine was added to

the reaction mixture to a final concentration of 10 mM. In par-

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crease in the reaction rate. This activation by L-aspartate was

observed when unlabeled L-glutamate was used as the substrate in

initial velocity was analyzed by varying the concentrations of

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served at L-glutamate concentrations above 20 mM, but no

such inhibition was seen with NAD. The apparent $K_m$ for NAD

determined to be 2.1 mM in the presence of 20 mM

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the presence of NAD, the $^1$H NMR spectrum of the proton at the C-4 position in the pyridine ring of the NADH produced showed detectable resonance peaks at 2.46, 2.51, 2.59, and 2.64 ppm (Fig. 5A). On the other hand, when deuterated dL-glutamate (2,4,4-d$_3$) was used as the substrate, the $^1$H NMR spectrum of the NADH produced showed a resonance peak at 2.58 ppm (Fig. 5B). This means that the enzyme reaction produced [4R-$^1$H, 4S-$^2$H]NADH from NAD and deuterated l-glutamate. Thus, *J. lividum* GDH exhibits pro-S specificity in its hydrogen transfer to NAD (B-type stereospecificity).

**DISCUSSION**

The first aim of the present study was to isolate psychrophiles and determine the characteristics of psychrophilic GDH. We succeeded in obtaining seven psychrophile strains that produced NAD- or NADP-dependent GDH. Of particular interest to us was strain No. 68. The mobility of the active GDH band detected when crude extract from this strain was run on a native PAGE gel was exceptionally low compared to the mobilities observed for the other strains. This suggested that the molecular mass of the GDH from this strain is much greater than those of the other GDHs, which prompted us to identify the strain (*J. lividum*) and to clone the gene and characterize the enzyme. As we anticipated, we found that *J. lividum* produces a very large NAD-dependent GDH, which has a homohexameric structure (1,065 kDa) with a subunit molecular mass of 170 kDa. Similar very large NAD-dependent GDHs with subunit molecular masses of around 180 kDa also have been found in *S. clavuligerus*, *P. aeruginosa*, and *Psychrobacter* sp. strain TAD1 (6, 17, 19). Interestingly, however, the GDHs from those three organisms exhibit hexameric, tetrameric, and dimeric structures, respectively. Judging from the subunit structure, we suggest that the hexameric *J. lividum* GDH belongs to the same group as the very large GDH from *S. clavuligerus* (1,100 kDa). On the other hand, the regulation of the catalytic activity of *J. lividum* GDH markedly differs from that of the *S. clavuligerus* enzyme and is more like the regulation of the *P. aeruginosa* and *Psychrobacter* GDHs. *S. clavuligerus* GDH is activated by AMP and is strongly inhibited by Tris
enzymes. Moreover, when we examined the sequence homology (60 to 65%) with the corresponding regions of these central domain (amino acid residues 719 to 1190) shows higher ever, within the amino acid sequence of J. lividum indicated by shading and asterisks, respectively. Residues that interact with L-glutamate and that are involved with the catalytic reaction are GDH-50. Sequences were aligned using Clustal W (24). Residues conserved in the three very large GDHs and in all the sequences are symbiosum, the enzyme’s activity by about 1,735% (Fig. 4). Although activation of J. lividum GDH does not appear to be affected by AMP, other nucleotides, or Tris.

One of the notable characteristics of J. lividum GDH is its marked activation by nonsubstrate amino acids, such as L-aspartate and L-arginine. In particular, L-aspartate enhanced the enzyme’s activity by about 1,735% (Fig. 4). Although activation by several amino acids, including L-aspartate, L-asparagine, and L-arginine, is also observed in S. clavuligerus and P. aeruginosa GDHs, the degree to which their activity is increased is substantially smaller (no more than 492% of the control value) than the degree seen with J. lividum GDH. In addition, the reported activation of the S. clavuligerus and P. aeruginosa GDHs occurred when the substrate concentrations were unsaturated, suggesting that the enhanced activity reflected increased affinity of the substrate for its binding site on the enzyme. By contrast, activation of J. lividum GDH occurred when the substrate concentration was saturated, which suggests that the activation reflected an increased in the maximum reaction velocity. Differences in the manner of activation and in the activator specificity between these GDHs may be associated with differences in the sequences of their N-terminal and/or C-terminal domains, as the homology between these domains is rather low. We are presently working to clarify the details of the relationship between the activation and the structure of this enzyme.

We found that the entire amino acid sequence of J. lividum GDH shows about 40% homology with the sequences of S. clavuligerus and P. aeruginosa GDHs, as well as those of putative GDHs from C. violaceum, R. metallidurans, S. putrefaciens, B. cenocepacia, X. campestris, P. atlantica, and T. fisca. However, within the amino acid sequence of J. lividum GDH, the central domain (amino acid residues 719 to 1190) shows higher homology (60 to 65%) with the corresponding regions of these enzymes. Moreover, when we examined the sequence alignment of the J. lividum GDH central domain and the Clostridium symbiosum GDH (Fig. 6), which is a homoxehamer comprised of 50-kDa subunits in which nine residues are known to be directly responsible for binding L-glutamate and expressing the catalytic activity (1, 23), six of the nine residues of C. symbiosum GDH, G376, V377, and S380 of the J. lividum enzyme. This motif is either GXGXXS or GXGXXA which are known to be responsible for the pro- S specificity for hydrogen transfer to NADH, are completely conserved in J. lividum GDH; however, G376 and V377, which interacts with the y-carboxyl, A163, which interacts hydrophobically with the side chain, and G164, which interacts with the amino group of L-glutamate, are replaced by arginine (R784), proline (P867), and valine (V868), respectively, in J. lividum GDH. In addition, the classical dinucleotide binding motif GXGXGXG is replaced by GXGXXS in the J. lividum enzyme. This motif is either GXGXXG or GXGXXA in the GDH-180 group. Upstream of this motif, T209, which interacts with the nicotinamide ring of NAD(P), and it is known that GDH-50s show B-type stereospecificity (16). Here we show for the first time that the very large J. lividum GDH shows the same B-type stereospecificity as GDH-50s (Fig. 5). These results suggest that the central domain of GDH-180s contains the active site where the enzymatic reaction occurs and that the central region of J. lividum GDH (amino acid residues 719 to 1190) strongly resembles that of C. symbiosum GDH. In addition, the N-terminal domain (amino acid residues 1 to 718) and C-terminal

FIG. 6. Amino acid sequence alignment of the central domains of P. aeruginosa, S. clavuligerus, and J. lividum GDHs (170 to 180 kDa) and C. symbiosum GDHs (170 to 180 kDa) and J. lividum GDH. In addition, the classical dinucleotide binding motif GXGXXG is replaced by GXGXXS in the J. lividum enzyme. This motif is either GXGXXG or GXGXXA in the GDH-180 group. Upstream of this motif, T209, which interacts with the nicotinamide ring of NAD(P), and it is known that GDH-50s show B-type stereospecificity (16). Here we show for the first time that the very large J. lividum GDH shows the same B-type stereospecificity as GDH-50s (Fig. 5). These results suggest that the central domain of GDH-180s contains the active site where the enzymatic reaction occurs and that the central region of J. lividum GDH (amino acid residues 719 to 1190) strongly resembles that of C. symbiosum GDH. In addition, the N-terminal domain (amino acid residues 1 to 718) and C-terminal
domain (amino acid residues 1191 to 1575) of I. lividum GDH may be responsible for regulating the enzyme activity, although their function is still unclear.

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