J. Microbiol. Biotechnol. (2000), **10**(5), 587–594

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Production and Characterization of Monoclonal Antibodies to Glutamate Dehydrogenase from Thermophile *Sulfolobus solfataricus*

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Received: March 22, 2000
Accepted: August 22, 2000

Abstract Monoclonal antibodies against glutamate dehydrogenase (GDH) from *Sulfolobus solfataricus* were produced and characterized using epitope mapping and biosensor technology. Five monoclonal antibodies raised against *S. solfataricus* GDH were each identified as a single protein band that comigrated with purified *S. solfataricus* GDH on the SDS-polyacrylamide gel electrophoresis and immunoblot. Epitope mapping analysis showed that only one subgroup among the antibodies tested recognized the same peptide fragments of GDH. Using the anti-*S. solfataricus* GDH antibodies as probes, the cross-reactivities of GDHs from various sources were investigated and it was found that the mammalian GDH is not immunologically related to *S. solfataricus* GDH. The structural differences between the microbial and mammalian GDHs were further investigated using biosensor technology (Pharmacia BIAcore) and monoclonal antibodies against *S. solfataricus* and bovine brain. The binding affinity of *S. solfataricus* glutamate dehydrogenase anti-*S. solfataricus* for GDH (K_D=11 nM) was much tighter than that of anti-bovine for GDH (K_D=450 nM). These results, together with the epitope mapping analysis, suggest that there may be structural differences between the two GDH species, in addition to their different biochemical properties.

Key words: Glutamate dehydrogenase, *Sulfolobus solfataricus*, monoclonal antibody, protein-protein interaction

Recently, living organisms have been classified into three primary kingdoms: eukaryotes, eubacteria, and archaebacteria [39]. The last of these is comprised of three different phenotypes: methanogens, extreme halophiles, and extreme thermophiles. While the glutamate dehydrogenases of eukaryotes and eubacteria have been well studied, the investigation of this enzyme from archaebacterial sources has been limited to the halophilic phenotype. The discovery of extremely thermophilic archaebacteria gave rise to a hope that the enzyme proteins of these organisms could provide structural requisites for thermophilic behavior more clearly than the proteins of rather moderately thermophilic eubateria and eukaryotes identified up until now. Accordingly, the enzymes from thermophilic bacteria are currently receiving much attention from researchers [21, 28, 42, 43]. These molecules may contribute to further understanding of the mechanisms of thermostability and their physicochemical properties may be particularly suitable to many biotechnological processes.

Sulfolobus solfataricus, a hyperthermophilic sulfur dependent microorganism living optimally at 89°C, is one of the well studied archaebacteria [24, 31] and a large biomass is easily obtained. Because *Sulfolobus* grows at very high temperatures, it should provide a source of enzymes with unusual physicochemical properties. In addition to its growth in extreme environments, the study of *S. solfataricus* as a member of the third primary kingdom of organisms [39] is interesting from a phylogenetic point of view. Since nitrogen metabolism of *S. solfataricus* is completely unknown [19], the abundance of glutamate dehydrogenase (GDH) and its putative involvement in amino group metabolism promoted us to study this enzyme. It has been previously shown that *S. solfataricus* GDH may be the first enzyme involved in the biosynthesis of an amino group by the conversion of 2-oxoglutarate and ammonia to glutamate [14].

Depending on the coenzyme involved in the reaction, GDH can be classified into two distinct classes [17]; the NADP-dependent enzyme which is involved in ammonia assimilation and the NAD-dependent one which takes part in glutamate catabolism. In a series of GDHs from different sources such as ox, chicken, and human liver [25], the

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NADP-dependent GDH showed a significant sequence similarity and similar hexameric structure with subunits of 45–56 kDa [35]. Based on this evidence, several authors have suggested similar conformations for these enzymes [4] and a common evolutionary origin [40]. On the other hand, other GDHs such as the NAD-dependent enzymes isolated from *Saccharomyces cerevisiae* and *Neurospora crassa* have a tetrameric assembly with subunits of 116 kDa. [38]. Despite of a large amount of information on several GDHs, there are still uncertainties regarding the catalytic mechanism and the role played by various effectors on the enzyme. It is only in recent years that the three-dimensional structure of GDH from microorganisms has been made available [3, 41]. More recently, crystallization of bovine liver GDH was reported [29]. There is, however, relatively little identity between microbial and mammalian GDHs, and comparison of the detailed structure and function of the various GDH species has rarely been reported.

In the present study, the production of monoclonal antibodies against the purified GDH from *S. solfataricus* was performed with the aim to study the structure-function and evolutionary relationships between various types of GDHs. The structural differences between microbial and mammalian GDHs were investigated further using biosensor technology (Pharmacia BIAcore) and monoclonal antibodies against *S. solfataricus* and bovine brain. The BIAcore system (Pharmacia Biosensor) allowed quantitative analysis of molecular interactions in real time. As a result, association and dissociation rate constants could be readily calculated. The present results suggest a possibility that there are structural differences in the epitopes of mammalian GDH and microbial GDH.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. solfataricus, strain MT-4, was purchased from ATCC, and was then grown aerobically at 87°C and pH 3.5, using a 0.2% yeast extract as the nutrient, as previously described [15]. The minimal growth media contained 1% casamino acid. The cells were harvested during the stationary growth phase by continuous shaking using a Lab-line orbit environ-shaker.

Purification of GDH from *S. solfataricus* and Enzyme Assay

S. solfataricus GDH was purified according to the procedure developed by us [2]. The enzyme activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in the absorbance at 340 nm [2]. All assays were performed in duplicate, and the initial velocity data were correlated with a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, and 2.6 mM EDTA at 60°C. The GDH concentrations were adjusted to give a measured rate of less than 0.04 absorbance units/min. The reaction was started with the addition of 2-oxoglutarate to a final concentration of 10 mM. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min at 60°C.

Production of Anti-GDH Monoclonal Antibodies

For injection, the purified GDH (50 μg in a volume of 150 μl) was mixed with an equal volume of Complete Freund's Adjuvant by sonication for three 15 sec bursts. The antigen-adjuvant mixture was injected into a female BALB/c mouse (8–10 weeks old). The first injection was followed by three booster injections at 3 to 4-week intervals. The final injection was given 3 days before the cell fusion without the adjuvant. The feeder layer cells were prepared one day before the fusion. The 16–18 week old BALB/c mouse was then killed by cervical dislocation, and its abdominal skin was carefully removed. Five milliliters of ice-cold 11.6% sucrose solution was injected into the peritoneal cavity, about 3 ml of the injected solution was pulled out, and the peritoneal cells were collected by centrifugation at 650 ×g for 5 min. The fusion experiments were performed as follows [13]. In brief, the spleen cells, released by tearing the removed spleen with fine forceps, were collected in a 15-ml centrifuge tube. Next, the collected spleen cells and SP2/o-Ag-14 mouse myeloma cells [34] were combined, and 1 ml of 50% polyethylene glycol 1500 in DME (serum free) was added slowly. The fusion process was allowed to continue for 90 sec at 37°C and then stopped by adding DME. To avoid an osmotic shock, 1 ml of DME was added slowly for the first 1 min, and 2 ml was added for the next 1 min. Over a period of 10 min, a total of 20 ml of DME was added. The cells were collected by centrifugation for 1 min at 650 ×g, suspended carefully in 20 ml of a selective hypoxanthine-aminopterin-thymidine medium (DME supplemented by 20% fetal bovine serum, antibiotics, and hypoxanthine-aminopterin-thymidine) by swirling, and centrifuged for 1 min at 650 ×g. The cells were resuspended in 120 ml of the hypoxanthine-aminopterin-thymidine medium, and 1 ml of the cell suspension was then transferred into each well of five 24-well plates. About 2 weeks after the fusion, the culture supernatants were collected and then screened first by an immunodot-blot analysis with the purified enzyme as the antigen and then by a Western blot analysis. The positive clones selected by the screening methods were transferred to six-well plates, grown in tissue culture flasks (75 cm^2), and frozen in liquid nitrogen tank. All the positive clones were first frozen and then cloned by limiting the dilution after thawing. For cloning a single specific antibody-secreting cell, aliquots of the cultured cells were diluted in a fresh

DME medium and counted using a hemocytometer. The samples to be cloned were diluted in an HT medium to 15 cells/ml. Seventy µl of the well-suspended sample were plated in each well of a 96-well plate to which 140 µl of fresh HT media were added. This was fed on day 5 and day 12 with two drops of medium. The cells of each well were expanded and reassayed by a Western blot analysis. The reaction of monoclonal antibodies with the bovine brain GDH was performed as previously described by the current authors [13].

Immunoblot Analysis

For immunodot-blotting, small squares (1×1 cm) were drawn on a sheet of nitrocellulose paper (10×10 cm) and marked by numbering. One microliter of antigen solution (0.5 mg/ml) was applied onto each square and air-dried. The blots were incubated for 1 h in Blotto [2% nonfat dry milk in Tris-buffered saline (TBS)], rinsed briefly with TBS, and air-dried. The blots were processed by the procedures as described in Western blotting. For Western blotting, the proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to a nitrocellulose membrane [37], and then the membranes were rinsed briefly in distilled water and air-dried. The blots were blocked with BLOTTO for 1 h. After rinsing with TBS, the blots were incubated in culture supernatants for 1 h and washed three times at 5 min intervals in TBS containing Tween-20. The blots were treated with alkaline phosphatase-conjugated goat anti-mouse IgG for 1 h and washed three times at 5 min intervals with TBS containing Tween-20. Following the final rinse for 5 min with an alkaline phosphatase buffer (100 mM Tris-HCl and 5 mM $MgCl_2$, pH 9.5), a color reaction was started by incubating the blots in an alkaline phosphatase buffer containing nitroblue tetrazolium and bromochloroindolyl phosphate. For 10 ml of solution, 66 µl of nitroblue tetrazolium (50 mg/ml in 70% dimethylformamide) and 33 µl of bromochloroindolyl phosphate (50 mg/ml in 100% dimethylformamide) were added to 10 ml of the alkaline phosphatase buffer. When the color reaction reached the desired intensity, the reaction was stopped by rinsing the membranes with several changes of distilled water. The blots were photographed while still moist.

Purification of Monoclonal Antibodies

For the purification of the monoclonal antibodies, 100 ml of the culture supernatant was centrifuged at 15,000 ×g for 30 min to clarify the cells and insoluble aggregates, and the supernatant was applied to 1 ml of a protein A-agarose column (Sigma). The column was washed with phosphate-buffered saline until the absorbance of the unbound proteins was reached to a background level, and then the antibodies were eluted with 0.1 M glycine-HCl, pH 2.5. The eluted antibodies were neutralized with the addition of 1 M Tris-HCl and dialyzed against phosphate-buffered saline.

Epitope Mapping

One-dimensional epitope mapping was carried out according to the procedure previously described [13]. Ten micrograms of purified *S. solfataricus* GDH in an SDS sample buffer were mixed with an equal volume of *Staphylococcus aureus* V-8 protease solution (0.5 mg in SDS sample buffer). The mixtures were applied to SDS-polyacrylamide gel and the separated peptides were transferred for an immunoblotting analysis, as described above.

Cross-Reactivities of the mAb Against *S. solfataricus* GDH with Mammalian GDHs and Other Microbial GDHs

Microorganisms were removed from several animals including dog, cat, cow, pig, and rat, and homogenized in 10 mM potassium phosphate containing 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM 2-mercaptoethanol. The individual 25% (w/v) homogenates were centrifuged at 10,000 ×g for 1 h, and 5 ml of each supernatant was mixed with an equal volume of a 2x SDS sample buffer and boiled for 3 min. The cooled samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Western blots were processed by the procedures described above.

Immobilization and Analysis of Proteins on BIAcore

The protein-protein interaction of *S. solfataricus* GDH with anti-*S. solfataricus* GDH and anti-bovine brain-GDH were performed using a Pharmacia Biosensor BIAcore instrument [13]. CM5 research grade sensor chips (Pharmacia Biosensor) were used for all experiments. The indirectly oriented immobilization of the antibodies on the CM5 sensor chip was carried out as follows. First, rabbit anti-mouse IgG Fc (ramfc) was coupled to the chip by injecting 100 ng of ramfc in 10 mM sodium acetate (pH 4.5) at a flow rate of 5 µl/min at 20°C. The carboxyl-methyl dextran matrix of the sensor chip was activated using a 30 µl (6 min) injection of a mixture of 0.2 M 1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide and 0.05 M *N*-hydroxysuccinimide in water to convert the carboxyl group of the sensor chip matrix to *N*-hydroxysuccinimide ester. This ester is susceptible to a nucleophilic attack by the amino groups of proteins, resulting in an amide linkage of the protein to the sensor chip. Under these conditions, typically 3,700 resonance units of ramfc were immobilized on the CM5 chip. The interactions of the two monoclonal antibodies from *S. solfataricus* and bovine brain with *S. solfataricus* GDH were measured by two subsequent injections; the monoclonal antibodies were captured by the ramfc, followed by the two different GDH antibody species. The protein-protein interaction studies were carried out in an HBS buffer (10 mM Hepes/KOH, pH 7.5, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20). The kinetic rate constants (k_{on} and k_{off}) and equilibrium dissociation

constants ($K_D=k_{off}/k_{on}$) were determined using BIAlogue Kinetics Evaluation Software.

RESULTS

Production of Anti-GDH Monoclonal Antibodies

The GDH purified according to the experimental procedure exhibited a single protein band on SDS polyacrylamide gel. To enhance the immunogenicity of the protein and obtain antibodies with a better reactivity on a Western blot, the purified enzyme was denatured in the presence of SDS and injected into animals. From two fusion experiments, 94 positive clones were initially screened by the immunodot-blot analysis. Because a goat anti-mouse IgG antibody was used as the second antibody, all the monoclonal antibodies screened by the procedure were from the IgG class. Among the hybridomas, some clones completely lost the ability to produce monoclonal antibodies or produced monoclonal antibodies that reacted weakly with the protein on the Western blots and thus were discarded. Twenty-five hybridomas of the 94 clones were finally selected for further study. Five representative monoclonal antibodies purified by the protein-A affinity column are shown in Fig. 1. To check the specificity of the anti-GDH monoclonal antibodies, the total proteins of *S. solfataricus* were extracted, separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the monoclonal antibodies. The antibodies specifically recognized a protein band corresponding to the position of the purified GDH on SDS-polyacrylamide gel (Fig. 2).

Epitope Mapping

The different immunoreactivities of the anti-GDH monoclonal antibodies with the GDH protein were further examined by an epitope mapping analysis with V-8 protease. The GDH was digested with V-8 protease and immunoblotted with anti-*S. solfataricus* GDH monoclonal antibodies. The results in Fig. 3 show that only one subgroup among the antibodies tested recognized the same peptide fragments of GDH. The monoclonal antibodies (gdhmAb1~gdhmAb5) showed

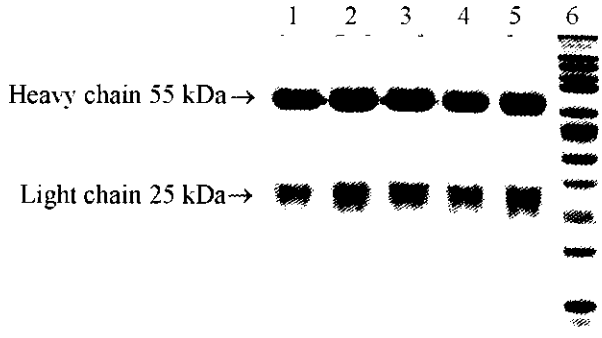

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified anti-GDH monoclonal antibodies.
A total of 25 anti-*S. solfataricus* GDH monoclonal antibodies was initially selected by immunodot-blot analysis and five representative antibodies purified by a protein-A agarose affinity column are shown here. Lane 1, gdhmAb1; Lane 2, gdhmAb2; Lane 3, gdhmAb3; Lane 4, gdhmAb4; Lane 5, gdhmAb5; Lane M, wide-range molecular weight marker proteins (Sigma).

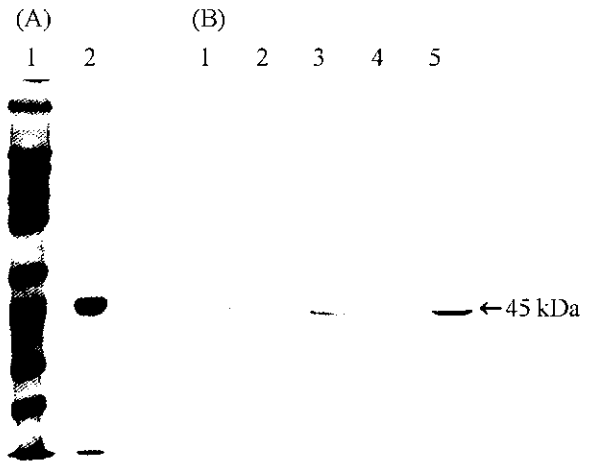

Fig. 2. SDS-polyacrylamide gel electrophoresis and corresponding immunoblots of purified *S. solfataricus* GDH probed with anti-*S. solfataricus* GDH monoclonal antibodies.
A. SDS-polyacrylamide gel electrophoresis. Lane 1, molecular weight marker proteins; Lane 2, purified protein of *S. solfataricus*. **B.** Corresponding immunoblots. Lane 1, gdhmAb1; Lane 2, gdhmAb2; Lane 3, gdhmAb3; Lane 4, gdhmAb4; Lane 5, gdhmAb5.

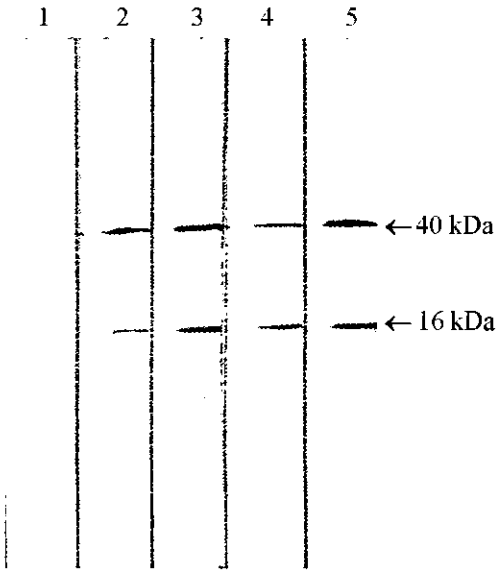

Fig. 3. Immunoreactivities of anti-*S. solfataricus* GDH monoclonal antibodies with *S. solfataricus* GDH digested with V-8 protease.
The purified GDH was digested with V-8 protease and separated on a 10–20% gradient SDS polyacrylamide gel. The separated peptides were transferred and immunoblotted with anti-*S. solfataricus* GDH monoclonal antibodies. Lane 1, gdhmAb1; Lane 2, gdhmAb2; Lane 3, gdhmAb3; Lane 4, gdhmAb4; Lane 5, gdhmAb5.

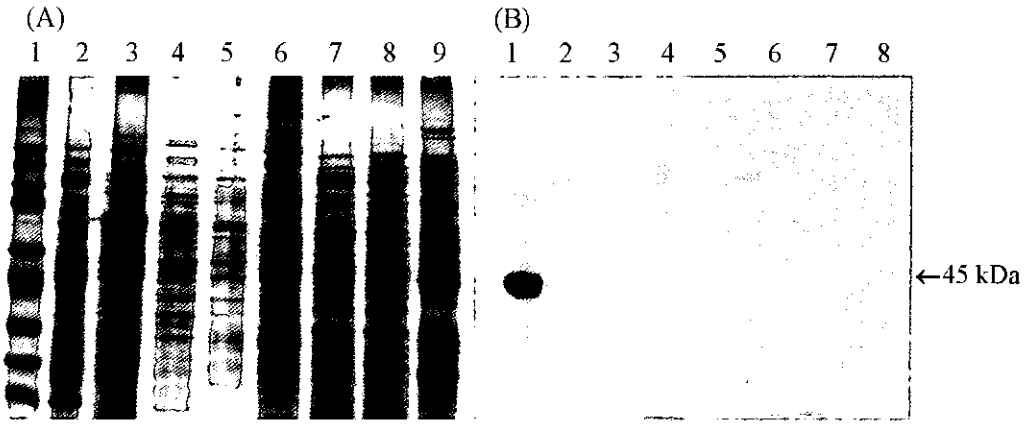

Fig. 4. Cross-reactivities of GDH from some other microorganisms with anti-*S. solfataricus* GDH monoclonal antibodies.
The total proteins of the homogenates were immunoblotted with the anti-*S. solfataricus* GDH monoclonal antibodies. The monoclonal antibodies (gdhmAb1~gdhmAb5) did not all recognize the GDH from each of the microbial species tested except for the GDH from *S. solfataricus*. Only the result with gdhmAb5 is shown for the sake of clarity. **A.** SDS-polyacrylamide gel electrophoresis of total proteins of each homogenate. Lane 1, molecular weight marker proteins; Lane 2, *S. solfataricus*; Lane 3, *Escherichia coli*; Lane 4, *Bacillus subtilis*; Lane 5, *Neisseria flava*; Lane 6, *Proteus vulgaris*; Lane 7, *Pseudomonas putita*; Lane 8, *Staphylococcus aureus*; Lane 9, *Streptococcus salivarius*. **B.** Corresponding immunoblot probed with monoclonal antibody (gdhmAb5). Lane 1, *S. solfataricus*; Lane 2, *Escherichia coli*; Lane 3, *Bacillus subtilis*; Lane 4, *Neisseria flava*; Lane 5, *Proteus vulgaris*; Lane 6, *Pseudomonas putita*; Lane 7, *Staphylococcus aureus*; Lane 8, *Streptococcus salivarius*.

two bands at 40 kDa and 16 kDa, indicating that the epitopes recognized by the five antibodies were either located nearby to one another or on the same site.

Cross-Reactivities of mAb Against *S. solfataricus* GDH with Mammalian GDHs and Other Microbial GDHs

The immunological relatedness of *S. solfataricus* GDH with enzymes from microorganisms was studied by immunoblotting the homogenate against various microorganisms. The homogenates from *Escherichia coli*, *Bacillus subtilis*, *Neisseria flava*, *Proteus vulgaris*, *Pseudomonas putita*, *Staphylococcus aureus*, and *Streptococcus salivarius* were prepared for the separation of their total proteins. The total proteins separated on SDS-polyacrylamide gel electrophoresis were transferred and probed with the five mAbs. The immunoreactive bands on the Western blot did not exhibit protein bands with the same molecular mass, 45–60 kDa, in all the microorganisms tested, and all five mAbs showed the same results (Fig. 4). The monoclonal antibodies reacted only with the GDH from *S. solfataricus*. These results suggest that there are striking differences between the microorganisms tested in the recognition site patterns of their monoclonal antibodies, having only 25–27% amino acid sequence similarities between *S. solfataricus* GDH and the other GDH species from *Saccharomyces cerevisiae*, *Neurospora cerevisiae*, and *Escherichia coli* [23]. Since structural information on the GDH species is not yet available, it was of interest to compare the immunoreactivities of the anti-*S. solfataricus* GDH monoclonal antibodies with different GDH species. In order to examine the cross-reactivity of the anti-GDH monoclonal antibodies with other mammalian and avian GDHs, homogenates from a dog, cat, cow, pig, and rat were removed and then the total proteins were separated, transferred, and probed with the 5 monoclonal antibodies. All five monoclonal antibodies did not recognize the GDH in each animal species tested (data not shown). These results on the cross-reactivities of GDH against various species suggest that *S. solfataricus* GDH is not immunologically related to mammalian GDHs.

Immobilization and Analysis of Proteins on BIAcore

To further compare the structural differences between *S. solfataricus* GDH and mammalian GDH, the immunoreactivities of *S. solfataricus* GDH with anti-*S. solfataricus* GDH and anti-bovine brain GDH monoclonal antibodies were quantitatively examined using Pharmacia BIAcore technology. The monoclonal antibodies used for this study were *S. solfataricus* gdhmAb5 and anti-bovine brain GDH [13]. Using the methods described above, k_{on} and k_{off} values were calculated for *S. solfataricus* gdhmAb5 and bovine brain gdhmAb. Each measurement was performed at least twice and up to four times on different surfaces. The results of the kinetic experiments are summarized in Table 1. The binding affinity of *S. solfataricus* GDH for the anti-*S. solfataricus* GDH monoclonal antibody (K_D=11 nM) was much tighter than for the anti-bovine brain GDH monoclonal antibody (K_D=450 nM). The difference between the anti-*S. solfataricus* GDH monoclonal antibody and the anti-bovine brain GDH monoclonal antibody in their binding affinity for *S. solfataricus* GDH was mainly caused by their association rate constants (k_{on}); 2.1×10^3 $M^{-1}s^{-1}$ and 1.6×10^2 $M^{-1}s^{-1}$ for the anti-*S. solfataricus* GDH monoclonal antibody and anti-bovine brain GDH monoclonal antibody, respectively. These results indicate that the molecular

Table 1. Interaction of *S. solfataricus* GDH with anti-*S. solfataricus* GDH monoclonal antibody and anti-bovine GDH monoclonal antibody.

S. solfataricus GDH	Association rate constant (k_{on})	Dissociation rate constant (k_{off})	Equilibrium constant (K_D)
	($M^{-1}s^{-1}$)	(s^{-1})	(nM)
S. solfataricus mAb	2.1×10^{3}	23.5×10^{-6}	11.2±0.3
Bovine brain mAb	1.6×10^{2}	72.1×10^{-6}	450.6±0.5

Results are the average of two separate experiments, with the error expressed as the range of the two data sets.

recognition process of *S. solfataricus* GDH for the antibodies against the *S. solfataricus* enzyme and that against bovine brain enzyme is quite different. It is, therefore, highly likely that the conformation of the protein epitope surface on *S. solfataricus* GDH is different from that of bovine GDH.

DISCUSSION

A number of different glutamate dehydrogenases from various sources have been identified. It is only recently that detailed information on the three-dimensional structure of any GDH has become available [36]. Despite of numerous studies on enzymes from thermophilic organisms mainly isolated from eubacteria, the structural principles which enable these enzymes to be stable and active at high temperatures remain unclear. In the present study, GDH from *S. solfataricus*, a thermophilic archaebacterium, was purified to homogeneity (Fig. 2A), and its main catalytic and structural properties were investigated. Interestingly, the coenzyme utilization of this enzyme resembled the equivalent enzyme from eukaryotes rather than eubacteria, since both enzymes recognized both NADH and NADPH with a high affinity. In general, mammalian GDHs have been known to be regulated by allosteric effectors such as ADP, GTP, and L-leucine [35]. The GDH from *S. solfataricus* was not regulated by GTP or ADP as in the case of GDH from *Neurospora crassa.* This enzyme contains 48 fewer amino acid residues than mammalian GDH, and there is little identity between the 100 residues in the carboxyl-terminal region [33]. It has been reported that *S. solfataricus* GDH contains 80 fewer residues than mammalian GDH [23]. It, therefore, seems likely that the regulatory binding domains of mammalian GDHs [7] are located in this region.

It should be noted that comparison of the amino acid sequence of *S. solfataricus* GDH with those from other species showed 25–27% identity, and a symmetrical evolutionary distance from the two groups of vertebrate on one side and eubacterial and low eukaryotes enzymes on the other side [23]. This seems to confirm the presence of different evolutionary pathways in the GDH class, probably due to the appearance of enzymes characterized by a less stringent requirement for the type of pyridine coenzyme and by no regulation of purine nucleotides and other reagents. A three-dimensional structural determination is obviously necessary to unequivocally determine the specific contribution of the amino acids to the regulatory process and to understand the nature of the enzymes.

In the present study, a library of monoclonal antibodies raised against *S. solfataricus* GDH was produced and the monoclonal antibodies were used for the first time to examine the structural relationship of the extreme thermophilic archaebacterium GDH and to compare it with those from other sources. From the immunoblotting analysis of the cross-reactivities test using the anti-GDH monoclonal antibodies with other GDH species, it is of interest to observe that a number of microbial GDHs and vertebrate GDHs were found to be immunologically different from *S. solfataricus* GDH. The results from the epitope mapping analysis (Fig. 4) support the possibility that the various GDH species tested could be different from *S. solfataricus* GDH, either in their amino acid sequences or protein structures. This also agrees with the hypothesis that archaebacteria evolved independently of the other groups, yet share a common ancestor [39]. However, it remains to be determined whether the fact that no cross-reaction of the anti-*S. solfataricus* GDH antibodies with the antigens tested was due to a different protein structure resulting from adaptation to a high temperature, or a consequence of the evolutionary distance of *S. solfataricus* as a member of the archaebacterial branch. To obtain more information from the epitope mapping analysis, an amino-terminal sequence analysis of *S. solfataricus* GDH is in progress in our laboratory.

The structural differences between microbial and mammalian GDHs were further investigated using biosensor technology (Pharmacia BIAcore) and monoclonal antibodies against *S. solfataricus* and bovine brain enzymes. The equilibrium binding constant between an antigen and an antibody can be measured in a variety of ways, as long as the complex can be separated from a free ligand once the reaction has reached equilibrium. However, few methods allow the analysis of the interaction in real time, thereby determining the kinetic rate constants. Biosensor technology uses the optical phenomenon of surface plasmon resonance to monitor the interaction of an immobilized ligand to a protein in the flow solution that is passed over it [16, 22]. The binding affinity of the anti-*S. solfataricus* GDH monoclonal antibody for *S. solfataricus* GDH was 40-fold tighter than that for bovine GDH (Table 1). These results indicate that the molecular recognition process of *S. solfataricus* GDH for anti-*S. solfataricus* GDH antibodies and that for anti-bovine GDH antibodies is different. It is, therefore, quite possible that there are differences between

S. solfataricus GDH and bovine GDH in their tertiary or quarterly structure, having only 25–27% amino acid sequence similarities between *S. solfataricus* GDH and mammalian GDH species such as human, ox, rat, and chicken [23, 27].

On the basis of specificity of monoclonal antibodies as characterized by a Western blot analysis, epitope mapping, and biosensor technology, the present results suggest that there are structural differences in the epitope between different GDH proteins. Taken together with the differences in their amino acid sequences [23] and in their regulatory properties [1, 8, 9, 10, 11, 12, 20, 32], the present findings support the possibility that different types of GDHs may function differently in a biological system, as many proteins have functions distinct from those for which they were originally identified. In fact, other roles of GDHs have been previously suggested. For instance, a membrane-bound form of GDH possesses a microtubule-binding activity [30] and GDH reacts as an RNA-binding protein with a possible role in the regulation of transcription [26, 5]. Recently, Cavallaro *et al.* [6] identified GDH as one of the late memory-related genes in the hippocampus and Frattini *et al.* [18] identified GDH as a new member of the ring finger gene family in Xq24-25. Accordingly, it would appear that unraveling of the mystery of GDHs and their role in the biological system has just begun.

So far, a comparison of the detailed structure and function of the various GDH species has rarely been reported. Therefore, further studies are required to elucidate the physiological roles of the various types of GDH proteins. One issue that was not addressed in this work was whether GDH proteins may provide the nitrogen metabolism in archaebacteria. This would offer new perspectives in understanding enzyme thermostability which may then reveal more information on the evolution of this enzyme during its progression towards an eukaryote. Furthermore, study on a possible evolutionary relatedness of *S. solfataricus* GDH with other well-known eukaryotic GDHs is also needed.

Acknowledgments

This work was supported by a grant from the Asan Institute for Life Sciences to S.-W. Cho and National Research Laboratory (NRL) grant (2000-N-NL-01-C-125) from the Korean Ministry of Science and Technology to S. Y. Choi.

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