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SCIENCE DIRECT
Gene 349 (2005) 237–244
GENE
SECTION
EVOLUTIONARY GENOMICS
www.elsevier.com/locate/gene

The discovery of four distinct glutamate dehydrogenase genes in a strain of *Halobacterium salinarum*

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Received 15 October 2004; received in revised form 9 December 2004; accepted 24 January 2005
Available online 17 March 2005
Received by T. Gojobori

Abstract

In earlier work, two glutamate dehydrogenase (GDH) proteins were purified from a strain of the halophilic archaeon *Halobacterium salinarum* (NRC-36014). One of these, an NAD^+-specific enzyme, was matched to a cloned gene from *H. salinarum* (GenBank accession number:X63837 S75579) by sequencing peptide fragments. Analysis of enzymatic digests of the $NADP^+$-GDH and database searching have now established that a gene encoding this protein exists in the full genomic sequence of *Halobacterium* sp. NRC-1 as *gdh*A1, together with two other distinct *gdh* genes, *gdh*A2 and *gdh*B. From N-terminal sequence, it is clear that the genomic listing incorrectly assigns the start codon for *gdh*A1 and the corresponding protein is 43 amino acids longer than previously indicated. The three genes could be amplified by PCR either from NRC-1, as expected, or from NRC-36014 (GenBank accession numbers: AY840085–AY840087). A gene encoding the previously purified NAD^+-GDH, is absent from the NRC-1 genome but can be successfully amplified from genomic DNA of NRC-36014 (GenBank accession number: AY840088). This establishes that NRC-36014 contains four *gdh* genes.

Keywords: Glutamate dehydrogenase; *Halobacterium salinarum*; Halophilic; Archaea; Gene sequence; Protein mass spectrometry; Degenerate PCR

1. Introduction

Glutamate dehydrogenases (GDH) are ubiquitous enzymes that catalyse the reversible oxidative deamination of L-glutamate to 2-oxoglutarate and ammonia using NAD^+ or $NADP^+$ as a cofactor (Frieden, 1963).

$$\text{Glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \longleftrightarrow 2\text{-oxoglutarate} + \text{NAD(P)H} + \text{NH}_4^+ + \text{H}^+$$

Individual GDHs may be NAD^+-specific (EC 1.4.1.2), $NADP^+$-specific (EC 1.4.1.4), or accept either coenzyme (EC 1.4.1.3). Many organisms have two or more GDHs of differing coenzyme specificity, usually associated with distinct metabolic roles, the NADPH-dependent enzyme being associated with assimilation of ammonia and the NAD^+-GDH with oxidation of glutamate.

Among the organisms reported to have more than one form of GDH is *Halobacterium salinarum* (Bonete et al., 1986, 1987), previously known as *Halobacterium halobium* (Ventosa and Oren, 1996). Initially, the separation and partial purification of two distinct forms of GDH were achieved from *H. salinarum* NRC-36014. These enzymes showed specificity for NAD^+ and $NADP^+$, respectively, and differed in size, thermostability, and other properties (Bonete et al., 1986, 1987). Subsequently, a *gdh* gene was cloned from a different strain of *H. salinarum* (CCM2090) (GenBank Accession Number X63837 S75579) and, on the basis of multiple alignments with other GDH sequences, predicted to encode an $NADP^+$-specific GDH (Benachenhou and Baldacci, 1991). This result was also used as the basis for a phylogenetic study (Benachenhou-Lahfa et al., 1993), which

Abbreviations: GDH, glutamate dehydrogenase [EC 1.4.1.2-4]; PCR, polymerase chain reaction; Asp-N, endoproteinase specific for cleavage at amino side of Asp residues [EC 3.4.24.33].

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doi:10.1016/j.gene.2005.01.011

must be of uncertain status as it overlooks the implications of the presence of paralogous genes in the same species.

It is now clear that molecular evolution has not cleanly separated distinct NAD^+-specific and $NADP^+$-specific arms in the GDH family, and that the switch in coenzyme specificity has occurred many times in the course of evolution (Lilley et al., 1991). Accordingly, attribution of coenzyme specificity purely on the grounds of sequence similarity to documented $NADP^+$-dependent GDHs is unlikely to be reliable. Our own more recent work with a purified NAD^+-specific GDH protein from *H. salinarum* NRC-36014 showed that a selection of its peptides exactly matched the published gene sequence for the enzyme reported (Benachenhou and Baldacci, 1991) as $NADP^+$-GDH of *H. salinarum* (CCM2090), suggesting that the assigned coenzyme specificity was wrong (Hayden et al., 2002). Reclonning of the published gene followed by over-expression and characterisation of its product showed unambiguously that this gene indeed encodes an NAD^+-specific GDH (Hayden et al., 2002).

The genome of *Halobacterium* sp. NRC-1 has now been sequenced (Ng et al., 2000) and indicates the presence of three GDH genes: *gdhA1*, *gdhA2*, and *gdhB*. None of these, however, matches the NAD^+-GDH previously sequenced from *H. salinarum* CCM2090 (Benachenhou and Baldacci, 1991) and NRC-36014 (Hayden et al., 2002), hereafter called *gdh*X.

A search for the authentic gene for the $NADP^+$-GDH of NRC-36014 and an attempt to fit the one *gdh* gene from this organism so far definitely associated with a functional enzyme (Hayden et al., 2002) into the overall pattern emerging through genome sequencing projects lead us now to report the presence of four distinct *gdh* genes in this strain of *H. salinarum*.

2. Materials and methods

2.1. Bacterial strains and reagents

Halobacterium sp. (NRC-1) and a colourless mutant of *H. salinarum* (NRC-36014) were generous gifts from Dr. W. F. Doolittle and Dr. M. J. Bonete, respectively. *H. salinarum* cells were grown at 37 °C in complex medium as described previously (Bonete et al., 1986). All chemicals used were of analytical grade. Coenzymes were purchased from Roche Biochemicals. The various salts used, Sepharose 4B and hydroxylapatite were supplied by Sigma. Restriction enzymes were obtained from New England Biolabs and *Pfu* polymerase was purchased from Stratagene.

2.2. Purification of $NADP^+$-GDH from H. salinarum (NRC-36014)

Preparation of cell-free extract and the purification procedure presented here are adapted from a previously published procedure (Bonete et al., 1986). Cells were harvested after 72 h growth in a shaking incubator. The buffer used was 0.05 M potassium phosphate, pH 6.6 (Buffer A), supplemented with varied concentrations of ammonium sulphate. A column (1.5 cm diameter) packed with 10 ml Sepharose 4B was equilibrated with Buffer A containing 2.5 M ammonium sulphate. The crude extract was loaded onto the column in the same solution, and the column was washed in turn with 1.5 volumes each of loading buffer, Buffer A with 2.0 M ammonium sulphate, and Buffer A with 1.8 M ammonium sulphate, all at a flow rate of 0.5 ml min^{-1}. Partially purified $NADP^+$-GDH eluted when the column was washed with 1.5 column volumes of Buffer A containing 1.6 M ammonium sulphate. The activity eluted as a more concentrated band if the flow was interrupted for 30 min soon after initiating the 1.6 M ammonium sulphate wash. No NAD^+-GDH activity was detected in the eluted fractions. The ammonium sulphate concentration of the pooled active fractions was adjusted to 2.5 M, and the protein solution was heated to 60 °C for 30 min. After the heat-step, the precipitate of thermolabile proteins was removed by centrifugation at 13000 rpm on a benchtop microcentrifuge and the supernatant was loaded onto a short, broad hydroxylapatite column (4.3 cm diameter, 0.7 cm height) previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.3, containing 4 M NaCl. The column was then washed overnight with about 10 column volumes of this phosphate buffer with a flow rate of less than 0.5 ml min^{-1}. A peristaltic pump led to compacting of the hydroxylapatite matrix and was therefore not used. The column was developed batchwise with 10 ml volumes of progressively increasing concentrations (0.05 M steps) of Na_2HPO_4 in 0.05 M potassium phosphate, 4 M NaCl, adjusted to final pH of 7.3. Fractions were collected and assayed for $NADP^+$-GDH activity.

2.3. SDS–PAGE analysis

SDS–PAGE was carried out on the partially purified $NADP^+$-GDH sample according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250, and protein bands were analysed with the Gel Documentation System (UVP).

2.4. Electroblotting of protein from SDS–PAGE and N-terminal sequencing

Polyvinylidenedifluoride membrane (Sequi-Blot, Bio-Rad) was soaked in HPLC grade methanol for 30 s, then placed in transfer buffer (20 mM Tris–HCl, pH 8.3, 150 mM glycine, 10% methanol) for 5–10 min. The gel containing proteins to be blotted was soaked in transfer buffer for 10–15 min. After electroblotting at constant current (220 mA) for 2 h, the membrane was rinsed with water (MilliQ) for 10–15 min, soaked in methanol (HPLC grade) for 10 s, and