Human aldolase A deficiency associated with a hemolytic anemia: Thermolabile aldolase due to a single base mutation

(DNA sequencing/expression vector/hereditary disease)

HIROYUKI KISHI†, TSUNEHIRO MUKAI*, AKIRA HIRONO†, HISAIICHI FUJII‡, SHIRO MIWA‡, AND KATSUMI HORII§

*Department of Biochemistry, Saga Medical School, Nabeshima, Saga 840-01, Japan; and †Department of Internal Medicine, Institute of Medical Science, Tokyo University, Tokyo 108, Japan

Communicated by Charles C. Richardson, July 20, 1987

ABSTRACT Fructose-1,6-bisphosphate aldolase A (fructose-bisphosphate aldolase; EC 4.1.2.13) deficiency is an autosomal recessive disorder associated with hereditary hemolytic anemia. To clarify the molecular mechanism of the deficiency at the nucleotide level, we have cloned aldolase A cDNA from a patient's poly(A)+ RNA that was expressed in cultured lymphoblastoid cells. Nucleotide analysis of the patient's aldolase A cDNA showed a substitution of a single nucleotide (adenine to guanine) at position 386 in a coding region. As a result, the 128th amino acid, aspartic acid, was replaced with glycine (GAT to GGT). Furthermore, change of the second letter of the aspartic acid codon extinguished a Fok I restriction site (GGATG to GGTG). Southern blot analysis of the genomic DNA showed the patient carried a homozygous mutation inherited from his parents. When compared with normal human aldolase A, the patient's enzyme from erythrocytes and from cultured lymphoblastoid cells was found to be highly thermolabile, suggesting that this mutation causes a functional defect of the enzyme. To further examine this possibility, the thermal stability of aldolase A of the patient and of a normal control, expressed in Escherichia coli using expression plasmids, was determined. The results of E. coli expression of the mutated aldolase A enzyme confirmed the thermolabile nature of the abnormal enzyme. The Asp-128 is conserved in aldolase A, B, and C of eukaryotes, including an insect, Drosophila, suggesting that the Asp-128 of the aldolase A protein is likely to be an amino acid residue with a crucial role in maintaining the correct spatial structure or in performing the catalytic function of the enzyme.

Fructose-1,6-bisphosphate aldolase (fructose-bisphosphate aldolase; EC 4.1.2.13) is a glycolytic enzyme that is composed of three distinct isozymic forms, aldolases A, B, and C (1). This enzyme has been studied extensively with respect to tissue distribution, changes during development, and carcinogenesis (1). It seems, therefore, that this enzyme is useful for studying molecular mechanisms of gene expression and also for understanding the evolution of the gene. In addition, elucidation of the molecular mechanism of aldolase deficiency is helpful for understanding the regulation of aldolase expression.

Genes for aldolases A and B have been cloned and characterized in rat (2–4), human (5–7), and other animals (8, 9). These results now permit us to study in detail the molecular basis of hereditary diseases caused by human aldolase deficiency. In inherited deficiency of aldolase in man there is a fructose intolerance, which is due to a liver aldolase B deficiency (9). Recently, another type of clinical entity, erythrocyte aldolase deficiency associated with hereditary hemolytic anemia, has been described (11, 12). Two cases (one kindred) of three were found in Japan. In these cases erythrocyte aldolase activity was very low and thermolabile, suggesting that the mutation is on the structural gene as opposed to being a mutation of gene regulation.

In the present paper we examine a case of erythrocyte aldolase deficiency and report that by comparing the nucleotide sequence of the patient's erythrocyte aldolase A cDNA with that of a normal control, an A-G transversion was found to occur in the codon for the 128th amino acid, aspartic acid (GAU). This results in the production of an enzyme having glycine (GGU) instead of aspartic acid. We also discuss the characteristics of the altered enzyme expressed in Escherichia coli.

MATERIALS AND METHODS

Materials. Reagents for measuring aldolase activity were obtained from Boehringer Mannheim. The cDNA synthesis system was from Amersham; the nitrocellulose filter was from Schleicher & Schuell; the nylon filter was from New England Nuclear; and [α-35S]dCTP (3704 Ci/mmol; 1 Ci = 37 GBq) was from ICN. Restriction enzymes and the other enzymes were from Nippon Gene and Takara Shuzo.

Cell Cultures. A lymphoblastoid cell line was established from a patient (Y.K.) with erythrocyte aldolase A deficiency (12) and from a normal volunteer by transforming the peripheral blood lymphocytes with Epstein–Barr virus. These cell lines were used in these studies because aldolase expressed in cultured lymphoblastoid cells was confirmed to be aldolase A (data not shown), the same type of enzyme as that expressed in erythrocytes (13).

Preparations of Aldolase A and Enzyme Assay. For electrophoresis, aldolase was partially purified from erythrocytes as follows. Cells that were briefly washed to remove the Buffy coat were disrupted and the lysates were passed through CM-Sephadex C-50 and fractionated by ammonium sulfate to remove hemoglobin. Aldolase activity was determined by two methods: activity staining (zymogram) and spectrophotometric methods. Electrophoresis on cellulose polyacetate strips and staining for aldolase activity were carried out as described by Susor et al. (14). Aldolase activity in the erythrocyte lysates was determined spectrophotometrically as described (15). The human aldolase activity expressed in E. coli was determined by the two methods described above in the presence of 5 mM EDTA to inhibit E. coli aldolase activity (16). To determine thermal stability of aldolase, cell lysates were incubated for 30 min at various temperatures in the presence of proteinase inhibitors—leupeptin, pepstatin,

Abbreviation: PhMeSO₂F, phenylmethylsulfonyl fluoride.

†Present address: Medical Institute of Bioregulation, Kyushu University, Fukuoka 812, Japan.
‡To whom reprint requests should be addressed.
from 1.10 to 50 units/g of Hb, respectively, supporting previous observation (12). The aldolase of erythrocytes and lymphoblastoid cells has the same defect as that of normal lymphoblastoid cells. Therefore, the patient’s aldolase in these cells was almost completely lost under the same conditions. These results indicate that the aldolase A expressed in the patient’s erythrocytes is much less stable than that in normal cells and also that aldolase A in the lymphoblastoid cell lines has the same defect as in the erythrocytes. The temperature sensitivity of the patient’s aldolase A suggested to us that the mutation probably occurred in the coding region of the aldolase A gene and that sequence analysis of the gene may be informative.

A Single Base Mutation Associated with Disappearance of a Restriction Site and an Amino Acid Substitution. cDNA clones were constructed from poly(A)+ RNA of the patient’s lymphoblastoid cell line using λgt10 as a vector and were screened using a rat aldolase A cDNA fragment as a probe (21). Ten clones positive for aldolase A were obtained from 20,000 plaques of cDNA library. One of the clones that appeared to carry the entire length of cDNA (pHAAdA5-2) was completely sequenced. The nucleotide sequence in the coding region of pHAAdA5-2 was the same as that of normal aldolase A cDNA (6) except for one nucleotide. In the patient’s cDNA clone, the 386th base from the ATG start codon, adenine, was replaced with guanine (Fig. 2). As a result, the 128th amino acid, aspartic acid (GAT), was replaced with glycine (GGT).

There are four Fok I recognition sites (GGATG) in normal human aldolase A cDNA that produce internal fragments of 33, 120, and 144 bp. One of the recognition sequences is located at the site including the 128th aspartic acid codon (GAT). Therefore, this single base change that occurs in the isoimyic form of the aldolase, the enzyme was subjected to zymography using the crude lysates of erythrocytes or lymphoblastoid cell lines from the patient and a healthy volunteer along with an authentic human aldolase A preparation. Aldolases in the normal control erythrocytes and the lymphoblastoid cell line migrated toward the anode along with the authentic enzyme (data not shown), indicating that the enzymes expressed in these human cells are of type A. Aldolase A activities in erythrocytes of the patient (Y.K.) and a normal control were 0.12 and 2.99 units/g of Hb, respectively, supporting previous observation (12). In the lymphoblastoid cell line of the patient and the normal control the aldolase A activities were 53.2 × 103 and 154.8 × 103 units/g of protein, respectively. These results indicate that the aldolase activity in the patient’s lymphoblastoid cells is also significantly lower than that of normal lymphoblastoid cells.

RESULTS
Thermolabile Aldolase Activity in a Patient with Hemolytic Anemia. Two cases (one kindred) of erythrocyte aldolase deficiency associated with hereditary hemolytic anemia have been reported in a Japanese family (12). The activity of aldolase in the patient’s erythrocytes was shown to be about 5% of that of normal and was thermolabile (12). To determine
patient's cDNA should be accompanied by the disappearance of this site and, therefore, results in the disappearance of one of the Fok I fragments. In fact, the 120-bp Fok I fragment disappeared in the patient's cDNA (data not shown), indicating that there was a base change in the GGATG of a Fok I site.

To verify that the base change in the patient's aldolase A cDNA was a consequence of the nucleotide substitution in the patient's genomic DNA, high molecular weight DNA (extracted from the patient's lymphoblastoid cell line) was digested with Fok I, separated on agarose gel, transferred to a nylon filter, and probed with the Fok I-Sau3A fragment of the normal human aldolase A cDNA (6) (Fig. 3A). If the Fok I site of the genomic DNA (corresponding to that of cDNA) was lost, then a 1-kilobase (kb) fragment should appear and a 0.3-kb fragment should disappear. As shown in Fig. 3B, the 0.3-kb fragment (labeled d in Fig. 3) completely disappeared and, instead, a 1-kb fragment (labeled a' in Fig. 3) appeared in the patient's DNA, whereas DNA from a normal lymphoblastoid cell line and genomic DNA cloned by us (T.M., H. Yatsuksi, K. Joh, Y. Arai, and K.H., unpublished data) gave a 0.3-kb fragment (Fig. 3B). The leukocyte DNA from the patient also gave essentially the same result (data not shown).

Thermal Stability of the Patient's Aldolase A Expressed in E. coli. To examine whether the adenine to guanine nucleotide substitution in genomic DNA resulted in the production of thermolabile aldolase A in the patient's erythrocytes, aldolase A cDNAs, prepared from normal control and patient's lymphoblastoid cell lines, were inserted into an E. coli expression vector that contained an lac promoter-operator (20). The expression vector was then transfected into E. coli strain JM83. The human aldolases, encoded for by normal and patient cDNAs and expressed in E. coli, were then used for a comparison of enzyme thermal stability (Fig. 4). Normal aldolase A retained about 70% of its activity after heat treatment at 50°C (pHA47), whereas the patient's aldolase A entirely lost its activity even at 40°C (pHADA524 and pHAD526). Thermal stability of the aldolases expressed in E. coli was quite similar to that of the enzymes from erythrocytes and lymphoblastoid cells, although the enzymes synthesized in E. coli were less stable.

When the Acc I fragment of pHDA526 was substituted with the corresponding Acc I fragment of pHA447, a normal aldolase A cDNA, the enzyme encoded for by the reconstituted plasmid (pHA471 and pHA473) displayed the thermal stability of the enzyme encoded for by the pHA447. These results indicated that a single base change of adenine to guanine at the 386th position was responsible for heat lability of the enzyme but that the remaining portion of the expression plasmid DNA was normal.

**DISCUSSION**

Three cases (two kindreds) of aldolase deficiency associated with congenital nonspherocytic hemolytic anemia have been described (11, 12). The present study presents an analysis of one of these cases that demonstrated the aldolase A defect at the nucleotide level. The nucleotide sequence analysis of the patient's aldolase A cDNA showed a substitution of the 386th base (adenine by guanine) in the coding region and the resultant replacement of aspartic acid, the 128th amino acid, by glycine. Using a system expressing human aldolase A in E. coli, the characteristic of temperature sensitivity of the aldolase A was reproduced in E. coli (Fig. 4). We clearly demonstrated that glycine instead of aspartic acid at the 128th position caused the aldolase A to be thermolabile.

Although there are many instances of inherited diseases in which the mutation of nucleotide sequence is identified (22), it is generally difficult to assess whether, if more than one mutation exists in a sequence, a particular mutation only represents a DNA polymorphism or causes a functional defect. However, our use of the E. coli expression vector in this study has proved to be very useful in surveying for mutations in coding sequences of genes that may influence the function of proteins. In addition, this method enables us to purify proteins from E. coli extract that contains the product encoded by E. coli expression plasmid and further to characterize the proteins biochemically.

Our study proves that the patient's aldolase A gene carries a homozygous mutation in the coding region of the genome since in Southern blot analysis cleavage at the relevant Fok I site did not occur in the patient's genomic DNA. The patient's parents carry the same mutation and are heterozygous for the mutant gene since they are phenotypically normal and have aldolase activities in erythrocytes that are
intermediate between normal and affected levels (12). These observations, together with the evidence that the aldolase A gene (ALDOA) is on chromosome 16 (23), support the idea that this hereditary disorder has an autosomal recessive mode of inheritance.

Vertebrate aldolases have three isozymic forms: A, B, and C. The amino acid sequences of aldolase A and aldolase B are highly conserved (24). The 128th amino acid, aspartic acid, found in normal human aldolase A is conserved in all aldolase isozymes so far examined, including human, rat, and rabbit A, human, rat, and chicken B, and rat and mouse C (25) and even in Drosophila aldolase (26). One of the reasons that the substitution of aspartic acid by glycine causes the thermostability of the enzyme may be due to the loss of a negative charge that is indispensable for retaining its conformational stability, especially at high temperature. It is thus possible that the aspartic acid fulfills an important role in the function of aldolase either by maintaining its structural stability or as a regulatory site.

The patient’s aldolase activity in erythrocytes was as low as about 5% of that of the normal control and was thermolabile. In the patient’s lymphoblastoid cell line the activity was about 30% of that of the normal control and also was thermolabile. We cannot explain at present why the remaining aldolase activities in lymphoblastoid cell line are higher than those in erythrocytes. Since the level of aldolase A mRNA in the patient’s lymphoblastoid cell line is usually higher than that in the normal control (data not shown), it is rather likely that large quantities of aldolase mRNA in the cultured cells partially compensate for its low activity, although we cannot eliminate other possibilities. Further studies are necessary to answer this question, including determination of the relative amount of aldolase mRNA in reticulocytes or lymphocytes of the patient.

We are grateful to Dr. M. Inouye of the State University of New York at Stony Brook for providing E. coli expression vector pN-III,

our colleagues for helpful discussions and comments, and Dr. Mark Bogart for reviewing the manuscript. This investigation was supported in part by Special Project Research Grant 60127010 (Inborn Errors of Metabolism) from the Ministry of Education, Science and Culture of Japan.