Molecular basis of human carbonic anhydrase II deficiency

(osteopetrosis/renal tubular acidosis/splice acceptor site mutation/missense mutation/compound heterozygote)

DONNA E. ROTH*, PATRICK J. VENTA†, RICHARD E. TASHIAN†, and WILLIAM S. SLY*†

*Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, MO 63104; and †Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109

Contributed by William S. Sly, November 21, 1991

ABSTRACT  Deficiency of carbonic anhydrase II (carbonate hydro-lyase, EC 4.2.1.1) is the primary defect in the syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification. In this report we describe the molecular basis for carbonic anhydrase II deficiency in the American family in which the association of carbonic anhydrase II deficiency with this syndrome was first recognized. The three affected siblings from this family are compound heterozygotes, each having inherited two different mutations in the structural gene for carbonic anhydrase II. The paternal mutation is a splice acceptor site mutation at the 3' end of intron 5. The maternal mutation is a missense mutation in exon 3 that substitutes a tyrosine for histidine-107. We show that the mutant enzyme expressed in bacteria from the cDNA containing the His-107→Tyr mutation has detectable, though greatly reduced, activity. We suggest that residual activity of the His-107→Tyr mutant enzyme may explain the absence of mental retardation and the relatively mild phenotype of carbonic anhydrase II deficiency in affected members of this family.

Osteopetrosis associated with renal tubular acidosis was first recognized as a distinct form of osteopetrosis in 1972. At that time, three independent families with this syndrome were reported from America, Belgium, and France (1–3). Family studies made it clear that this disease was recessively inherited (4). In 1983, Sly et al. (5) presented evidence that deficiency in carbonic anhydrase II (CA II; carbonate hydro-lyase, EC 4.2.1.1) was the primary defect in this syndrome. This enzyme catalyzes the reversible hydration of CO2 for review, see ref. 6). A modification of Maren’s end-point titration of CA activity (7) provided a simple assay for diagnosis of CA II-deficient patients and for identification of asymptomatic carriers (8).

More than 40 CA II-deficient patients from a wide variety of ethnic origins have been diagnosed (9, 10). Although the clinical features of the disease vary in severity among the different families, most patients present with multiple bone fractures, renal tubular acidosis, and mental retardation. The American family studied here is one of two families described in which the affected patients have the osseous and renal manifestations of the disease but lack the mental retardation (2, 4, 11).

The cDNA for the human CA II gene was isolated and sequenced in 1987 (12, 13), and the genomic organization was recently reported (14). This information allowed us to develop a general strategy for analysis of the CA II gene from CA II-deficient patients. Application of this strategy to the first American family reported with this syndrome (1, 11) revealed that the three affected siblings from this family are compound heterozygotes. They have inherited a splice acceptor site mutation from their father and a missense mutation in exon 3 [His-107→Tyr, which we refer to in single letter code (H107Y)] from their mother. The H107Y mutant enzyme, when expressed in bacteria, had a tendency to form insoluble aggregates, but some residual catalytic activity could be demonstrated in cells induced at 30°C and 20°C.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) and Sequencing. Exons of the CA II gene were specifically amplified by the PCR with 1 μg of genomic DNA, 50 pmol of each primer (Fig. 1), standard PCR buffer (50 mM KCl/10 mM Tris chloride, pH 8.3/1.5 mM MgCl2/0.01% (wt/vol) gelatin), 200 μM of dNTPs, and 2.5 units of Taq DNA polymerase (Perkin–Elmer/Cetus) in a total volume of 100 μl (15). The reaction for exons 2 through 7b consisted of 30 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 2–3 min) followed by a final 10-min extension at 72°C. Amplification of exon 1 required different conditions: 2.5 units of Taq DNA polymerase was added twice, once at the beginning and once after 15 cycles; 0.05 mM tetramethylammonium chloride was added to the standard PCR buffer (16); and 7-deaza-2′-deoxyxanosine 5′- triphosphate (dC‘GTP) and dGTP were added at a ratio of 3:1 (respectively 150 μM and 50 μM) (17). PCR conditions for exon 1 included denaturation at 96°C for 10 min, 35 cycles (96°C for 1 min, 60°C for 2 min, and 72°C for 3 min), and a final 10 min extension at 72°C. Primer pairs designed for amplification of each exon are listed in Fig. 1. All but one of the primers have an additional 12 bases, which include a 6-base restriction site, a 2-base spacer, and a 4-base cap. One-tenth of each PCR sample was analyzed by electrophoresis through a 1.2% agarose gel stained with ethidium bromide.

For sequencing, PCR products were isolated in 1% low-melting agarose gels (SeaPlaque; Midwest Scientific, St. Louis), cut with the appropriate restriction enzymes, and ligated into phage M13 sequencing vectors (mp18 and mp19 from Pharmacia) as described by Maniatis et al. (18). Clones were sequenced by the dideoxynucleotide chain-termination method of Sanger et al. (19) with [α-35S]dATP and T7 DNA polymerase (Sequenase; United States Biochemical). At least two separately amplified PCR products were cloned, and a minimum of 10 clones were sequenced for each exon.

Allele-Specific Oligonucleotide Analysis. One microgram of genomic DNA was amplified by PCR, extracted with 1:1 (vol/vol) phenol/chloroform, precipitated with ethanol, and resuspended in 50 μl of water; 350 μl of 0.4 M NaOH/0.025 M EDTA was added to each sample. This mixture was applied in duplicate (200 μl each) onto Nytran filters (Schleicher & Schuell) under vacuum by using the Hybridot manifold apparatus from BRL. For use as probes, 15-mer allele-specific oligonucleotides (Fig. 3) were end-labeled with [γ-32P]ATP

Abbreviations: CA II, carbonic anhydrase II; H107Y, His-107→Tyr mutation.
1To whom reprint requests should be addressed at: Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
FIG. 1. PCR primers for analysis of CA II mutations. Shown are the forward and reverse primers for each exon. The exon amplified is indicated on the left. The size of the PCR product is given in base pairs (bp).

(18). The filters were hybridized with these two allele-specific probes by using 3 M tetramethylammonium chloride in prehybridization, hybridization, and wash buffers (20).

**Acc I Restriction Site Analysis.** One-twentieth of each PCR sample was digested with the restriction enzyme Acc I (Promega) at 37°C for 2 hr. Products were analyzed by electrophoresis through 1.2% agarose gels stained with ethidium bromide.

**Bacterial Expression of the H107Y Mutant.** The full-length cDNA for CA II, isolated in this laboratory (12), was modified by site-directed mutagenesis in M13 to create an Nco I site at the ATG start codon (pDRM12). This changed the second amino acid from serine to alanine, which had no effect on enzyme expression when subcloned into bacterial or eukaryotic expression vectors. Carol Fierke (Duke University) subcloned the Neo I/Bgl II fragment of this construct, which contains all of the coding sequences, into the Neo I/BamHI sites of the Escherichia coli expression vector pET-8c (21) and supplied it to us as pCA (22). pCA was digested with EcoRV and Ssp I, and the blunt ends were ligated to remove the vector HindIII restriction site (pCAH3). The H107Y mutation was created in the modified CA II cDNA cloned in an M13 vector (pDRM12, described above) by site-directed mutagenesis with the Amersham oligonucleotide mutagenesis kit. The single-stranded DNAs from several clones were sequenced from the Neo I site to the HindIII site to ensure that no other mutations were introduced. The Neo I/HindIII fragment containing this mutation was isolated and ligated into pCAH3 from which the normal Neo I/HindIII fragment had been removed (pCAH3-H107Y). All manipulations of DNA were as described by Maniatis et al. (18).

pCAH3 and pCAH3-H107Y mutant clones were transformed into BL21(DE3)pLysS cells and induced with 0.6 mM isopropyl β-D-thiogalactopyranoside (21). Cells were pelleted, and a crude lysate was prepared by adding Triton X-100 to 0.1%, followed by freeze/thaw cycles. CA II activity was assayed in crude lysates centrifuged at 14,000 × g for 5 min by the end-point titration method (8). SDS electrophoresis was carried out as described by Laemmli (23), followed by electrophoretic transfer (Milliblot/SDS blotto; Millipore) and immunostaining as described by Zhu and Sly (24). The antibody to CA II was produced in rabbits immunized with recombinant human CA II. The recombinant human CA II protein was produced in E. coli by using the prokaryotic expression vector pKK232-2 containing the Neo I/EcoRI fragment from pDRM12 described above. The recombinant human CA II protein was purified on an inhibitor affinity column (25). Goat anti-rabbit IgG-peroxidase conjugate was used as a second antibody.

**RESULTS**

**PCR Analysis for Each Exon of CA II.** Venta et al. (14) recently described the genomic organization of the human CA II gene. Using this information and sequencing the exons 3 and 4 that were determined independently, we designed PCR primers flanking each of the seven exons of the CA II gene. Fig. 1 lists the primer pair for each exon amplified and the size of the PCR product. Fig. 2A shows the genomic organization of the CA II gene and the regions amplified for this analysis. Each exon of the CA II gene was amplified from genomic DNA from either a normal control or from one of the affected siblings from the American family first reported in 1972 (1, 11). Patient and control PCR products were electrophoresed on 1.2% agarose gels. Fig. 2B shows that the patient has a band for each exon that is comparable in size to the band from the control.

**Mutational Analysis of the CA II Gene. Paternal mutation.** Each of the exons were amplified from patient and control DNA, and every exon was sequenced. Fig. 3, which shows the partial sequence of the amplified products including exon 6, reveals a G-to-C base change in the splice acceptor of intron 5 in the patient DNA. The G → C transversion in the

![Fig. 2. (A) Genomic organization of the 20 kilobase CA II gene showing exons and strategy for analysis of exons by PCR. Bars E1 through E7b represent PCR fragments and show the regions of the CA II gene covered by this analysis. (B) Agarose gel (1.2%) showing the results of amplification of genomic DNA using the PCR primers listed in Fig. 1. The exon represented by each pair of lanes is indicated above and corresponds to the respective bar in A. Lanes: N, DNA from a normal control; P, DNA from patient D.C.; the youngest of three affected siblings shown in the pedigree (Fig. 4). kb, Kilobase ladder molecular weight standard (BRL).]
A-G splice acceptor dinucleotide would be expected to disrupt proper splicing of exon 6 (26, 27). This alteration is found in 50% of the patient’s clones (5 of 10), suggesting that it is present on only one of the patient’s two alleles.

Exon 6 from both parents was analyzed in a similar fashion. All of the mother’s clones were normal in the region of exon 6 (9 of 9 clones). However, 50% (5 of 10) of the clones from the father’s amplified exon 6 had the G → C base substitution, and 50% had the normal sequence. Thus, the father has one allele carrying the splice junction mutation at the intron 5 acceptor site and one normal allele. The analysis identified the paternal mutation and indicated that the paternal mutation must lie in another exon.

Dot-blot hybridization using allele-specific oligonucleotide probes was used to identify the paternal mutant allele in other members of the family (Fig. 4). Amplified DNAs from all three affected siblings (III-1,3,4), the father (II-1), the father’s two heterozygous siblings (II-1,5,6), and the grandfather (I-1) all hybridized with the mutant allele-specific oligonucleotide probe. This analysis demonstrated a complete correlation between half-normal levels of enzyme and the presence of this mutant allele in the paternal side of the family. No relative with normal CA II levels was found to have the mutant allele. All subjects tested hybridized to the normal allele-specific oligonucleotide, indicating a normal allele for this exon in all of the carriers of the paternal mutation and one normal allele (at this site) in the three CA II-deficient patients.

Maternal mutation. Fig. 5 shows the partial sequence of the amplified products including exon 3 and reveals a missense mutation in exon 3 of this patient. The C → T transition causes the histidine at position 107 to be replaced by a tyrosine (H107Y mutation). This missense mutation was found in 50% of the patient’s clones for this region.

The C → T mutation introduces a new Acc I restriction site in the mutant allele. The normal 935-bp PCR product for exon 3 contains a single Acc I site upstream of the coding region which, upon digestion, results in fragments of 800 bp and 135 bp. Digestion of a PCR product from a patient homozygous for this mutation with Acc I cleaves the 800-bp fragment into 620- and 180-bp fragments. Fig. 6 illustrates the use of the new Acc I restriction site to ascertain the presence or absence of this mutation within the immediate family. The two unrelated spouses of II-3 (II-2 and II-4) and the father (I-1) had only the 800-bp fragment corresponding to normal alleles for exon 3. The mother (I-2), the three affected siblings (II-1,5,6), and their heterozygous sister (II-3) had both a normal exon 3 (800-bp fragment) and a mutant exon 3 containing the new Acc I site (620-bp fragment). The two daughters (III-1,2) of the heterozygous sibling (II-3) were previously classified as “carriers” having half-normal levels of enzyme activity (5). Presence of the new Acc I site showed that their reduced enzyme level correlates with the presence of the allele carrying the maternal H107Y mutation.

Expression of the H107Y Mutant in E. coli. E. coli BL21(DE3)pLysS cells were transformed with prokaryotic expression vectors containing either the normal CA II cDNA (pCAH3) or cDNA containing the H107Y mutation (pCAH3-H107Y). Fig. 7 shows the normal and mutant CA II isozymes in total lysates from bacteria induced at 30°C and 37°C. At both temperatures, the 29-kDa CA II is the predominant protein seen on the Coomassie-stained PAGE gel (Fig. 7A). The Western blot confirms that this protein is CA II (Fig. 7B). The mutant protein is present at 20–25% of the level of the normal CA at both temperatures.

Fig. 3. Sequence of PCR fragments from both normal and patient DNA showing the region surrounding the junction of intron 5 and exon 6. The arrows indicate the base at which the mutation occurs (G → C).

Fig. 4. (Upper) Rows I, II, and III show the pedigree used in this analysis. The family members are scored for erythrocyte CA II isozyme levels and activity (5, 8). Solid figures represent the affected patients who have no detectable erythrocyte CA II protein or activity. Half-filled shapes have intermediate levels (heterozygotes), and open figures have normal levels of CA II protein and activity. (Lower) Allele-specific oligonucleotide analysis of the paternal mutation. DNA from the family members was applied to nylon filters in duplicate. One filter was hybridized with a 32P-labeled normal allele-specific probe (CA2X6D*: 5'-TGTTCTAGGCACAGA-3'). This result is shown in the row labeled G*. The duplicate filter was similarly hybridized with a 32P-labeled mutant allele-specific probe (CA2X6D*: 5'-TGTTCTACGGCAAGA-3'). This result is shown in the row labeled C*D.

Fig. 5. Partial sequence of PCR fragments including exon 3 from normal and patient DNA. The amino acids specified by each codon are indicated. The arrows point to the base substitution (C → T) in the patient’s codon for His-107 that leads to replacement of the histidine at position 107 by a tyrosine residue.
normal levels of CA II in erythrocytes from their obligately heterozygous parents suggested that a mutation in the structural gene for CA II was the molecular defect in this disorder (5). The family presented here is the second for which a structural gene mutation has been demonstrated (14). The three affected siblings are compound heterozygotes, having inherited a splice acceptor site mutation from their father and a missense mutation from their mother.

The maternal mutation (G → C) disrupts the normal A-G dinucleotide in the splice acceptor site of intron 5. All heterozygotes who had this mutation had half-normal CA II activity in their erythrocyte lysates, and the three compound heterozygotes had no detectable activity. Loss of the splice acceptor site in intron 5 would be expected to cause the skipping of exon 6, the use of a cryptic splice site, or retention of intron 5 (26, 27). Any one of these could explain the CA II deficiency, since exon 6 contains codons for 5 of the 17 invariant amino acids that are thought to be involved in the active site of CAs (6). If RNA from the father’s reticulocytes could be obtained, one might demonstrate multiple mRNAs for CA II, one corresponding to the transcribed product of the normal allele and the other(s) to product(s) of the aberrant splicing predicted from the mutation.

The maternal mutation (H107Y) in this family is the same mutation for which the original Belgian patient was recently shown to be homozygous (14). Consanguinity in the Belgian pedigree explained the homozygosity in the affected offspring. The Belgian patient (whose intelligence was also normal) had no measurable activity in erythrocyte lysates, and no enzymatic or immunoreactive activity was detected in lysates of bacteria expressing the mutant cDNA. In that report, molecular graphic comparisons of the H107Y mutant protein and normal CA II were presented that suggest that the loss of critical hydrogen bonds between His-107 and Glu-117 and between His-107 and Tyr-194 could explain the instability of the H107Y enzyme. Failure to observe the H107Y mutant protein at 37°C in that study probably reflects the tendency of the H107Y mutant enzyme expressed at 37°C to form insoluble aggregates.

Our expression data clearly showed that the H107Y mutant enzyme is expressed in bacteria. Although the activity of the mutant protein expressed in bacteria was drastically reduced, compared to that of the normal enzyme, residual activity could easily be demonstrated in cells induced at 30°C and 20°C where a larger fraction of the expressed enzyme remained soluble (80% at 20°C).

The residual activity of the H107Y mutant enzyme may explain the relatively mild phenotype and absence of mental retardation in the Belgian patient, who is homozygous for this mutation, and the patients in the family reported here, who are compound heterozygotes for this mutation and the splice acceptor site mutation. In their case, however, it is theoretically possible that the splice acceptor site mutation could also contribute to a milder phenotype. For example, an alternatively spliced mRNA might encode an abnormal enzyme with some residual activity. Although no residual activity was measurable in erythrocytes from patients in this family, residual activity could be present in nucleated cell types that express CA II.

In any case, the data presented provide clear evidence that the molecular basis for CA II deficiency in patients from this family is a combination of two different mutational alterations, one in each of the two alleles of the structural gene for CA II. The methods presented can be used for DNA-based diagnosis and counseling of other patients with these specific mutations, as well as to analyze CA II gene defects in families with other mutant alleles.

We thank Dr. Hiroshi Murakami for providing the original full-length cDNA clone for CA II, Dr. Carol Fierke for the pET
expression vector containing the coding sequence of CA II, and Dr. Abdul Waheed and Mr. Jeffrey Grubb for instruction, guidance, and inspiration. This study was supported by Grants GM34182 and DK40163 from the National Institutes of Health. D.E.R. was supported by Fellowship 18-87-08 from the March of Dimes.