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Carbonic Anhydrase II Deficiency: Diagnosis and Carrier Detection Using Differential Enzyme Inhibition and Inactivation

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SUMMARY

Carbonic anhydrase (CA) I and II are soluble isozymes that represent the major nonhemoglobin proteins in the erythrocyte. We recently identified a deficiency of CA II as the enzymatic basis for the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. Virtual absence of the CA II peak on high-performance liquid chromatography, of CA II esterase activity, and of immunoprecipitable CA II were demonstrated on extracts of red cell lysates from all patients studied. Reduced levels of CA II were found in obligate heterozygotes. Here, we present evidence that CA II in red cell lysates can be quantitated by measuring CO_2 hydratase activity in the presence of inhibitors that selectively inhibit the activity of CA I to a much greater extent than that of CA II. This was done with iodide (anion binding) and bromopyruvic acid (alkylation), and the respective assays evaluated as diagnostic tools for CA II deficiency in human red cells.

These techniques greatly simplify the quantitation of CA II in hemolysates and should make genetic diagnosis and counseling for the newly described inborn error of metabolism due to CA II deficiency generally available. They also allow quantitation of CA I in red cell lysates.

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INTRODUCTION

Carbonic anhydrase (CA) exists in three known soluble forms in man [1, 2]. All three isozymes (CA I, CA II, and CA III) are monomeric, zinc metalloenzymes with a molecular weight approximating 29,000. The enzymes catalyze the reversible hydration of CO_2 in reaction I:

$$\underset{\text{I}}{CO_2 + H_2O} \rightleftarrows \underset{\text{II}}{H_2CO_3} \rightleftarrows HCO_3^- + H^+ .$$

Reaction II is ionic, virtually instantaneous, and not subject to enzymatic acceleration. Genetic evidence suggests that the soluble CA isozymes are specified by members of a multilocus gene family derived by gene duplication from a common ancestral gene [2]. Each isozyme has characteristic properties and tissue distribution. CA I and CA II are found in appreciable amounts in human erythrocytes (11.6 ± 2.3 and 1.8 ± 0.3 [mean ± SD] mg/gm Hb, respectively) [1]. CA III, which is abundant in muscle [3], is present in lower amounts in adult red blood cells (147 ± 7 [mean ± SD] μg/gm Hb) [4, 5]. A membrane-bound form of carbonic anhydrase, tentatively designated CA IV, is known to exist in the lung and renal parenchyma [6–9].

The isolated deficiency of CA I appears to have no clinical consequences in man [10]. Deficiency of CA II produces the autosomal recessive disorder of osteopetrosis with renal tubular acidosis and cerebral calcification [11]. This disorder was initially described in three independent reports in 1972 [12–14]. In 1983, CA II deficiency was documented in three affected members of one family. Subsequently, 18 similarly affected patients from 11 unrelated families of varied geographic and ethnic origin have been studied. This second study confirmed the generality of the original findings and supported the conclusion that CA II is the enzymatic basis for this autosomal recessive syndrome [15, 16]. Heterozygotes for CA II deficiency are clinically asymptomatic but can be detected by reduced CA II levels in erythrocyte lysates using high-performance liquid chromatography (HPLC) [17]. This assay is not widely available and requires careful standardization. In addition, it has some limitations when samples are obtained from areas distant to the testing site, being reliable only on unfrozen samples assayed immediately following preparation of hemolysates.

Maren and Couto [18] reported that the CO_2 hydratase activity of CA I was nearly 100× as sensitive to inhibition by sodium iodide as that of CA II. Subsequently, Goethe and Nyman [19] demonstrated that CA I was also much more sensitive than CA II to inactivation by bromopyruvic acid. It was suggested to us by Drs. T. Maren and C. Conroy that these differences between CA I and CA II [18–22] might allow us to develop a simpler clinical assay for CA II in hemolysates. Here, we present studies of the CO_2 hydratase activity in red cell lysates of control subjects and of patients with CA II deficiency from two families. These studies are consistent with the prior reports of relative resistance of human CA II to inhibition by sodium iodide and to inactivation by bromopyruvic acid and suggest conditions for a clinically useful assay for CA II deficiency.

MATERIALS AND METHODS

Supplies and Materials

A 2.5-gallon glass fish tank or other transparent ice-glass container is used for the assay. CO_2 is delivered via a CO_2 flow meter (Cole-Palmer, Chicago, Ill., S.G.-3217-26) to ensure constancy of flow. Pasteur pipettes, micropipettes, and a supply of 10 × 100-mm glass tubes are required. All reagents were obtained from Sigma, St. Louis, Mo., with the exception of Octanol, which was from Fisher, St. Louis, Mo. Five reagents, prepared as described, are required: (1) Phenol red was made in a concentration of 12.5 mg/l distilled H_2O and stored refrigerated. (2) Barbital buffer, pH 7.9, was prepared from 4.6 g diethylbarbituric acid and 5.16 g of the sodium salt of diethylbarbituric acid and dissolved in 1 liter of heated distilled water. Barbital buffer is best stored at room temperature, since prolonged refrigeration causes recrystallization. (3) Sodium iodide was made up as a stock solution of 125 mM. (4) Bromopyruvic acid (40 mM) was freshly prepared each day; 66.8 mg of the acid is dissolved in 9.0 ml of 100 mM sodium phosphate, pH 6.8, the pH corrected with 1 M sodium hydroxide, and volume readjusted to 10 ml total with 100 mM sodium buffer, pH 6.8. (5) Octanol (primary) one bottle.

Preparation and Storage of Hemolysate

Whole blood was collected from 22 healthy donors and from two families with members affected by the syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. The blood was collected in lithium heparin tubes and stored on ice until diluted. Dilutions (1:100) were made with 0.05% Triton X-100 in distilled water (Triton is not required). Diluted samples were kept frozen at −70°C and thawed prior to use. Samples from pedigree B were sent on wet ice by air carrier from Italy and were already hemolyzed on arrival. Dilutions were made on arrival as described above and samples frozen at −70°C.

Hemoglobin Estimation

A 1:100 dilution of hemolysate in .05% Triton X-100 was used. Two hundred microliters of the 1:100 dilution was added to 800 μl of 0.006 NH_4OH, and the optical density read at 576. Hemoglobin (Hb) concentration of the sample was calculated from the formula: Hb (mg/ml) = (OD576 × 5 × 200)/0.912.

Procedure

The assay is based on that described by Maren [20]. The fish tank or comparable glass container is filled three-quarters with ice chips. All solutions are kept on ice for approximately 30 min prior to use. Reactions are carried out at 0°C. A test tube with 4 ml of phenol red in distilled water is placed against the front glass of the tank, next to a capped glass tube containing the pH standard (we used 4 ml of phenol red in distilled water and 2 ml of 50 mM phosphate, pH 7.2). Two to three drops of octanol were added to each test sample to reduce foaming. CO_2 is bubbled continuously at 600 ml/min through a Pasteur pipette to saturate the test solution. After 60 seconds of uninterrupted CO_2 flow, 100 μl of diluted enzyme is added, followed by 2 ml of barbital buffer rapidly added and timing by stopwatch begun. The reaction reaches end point when the indicator color matches that of the 7.2 pH standard. It is essential that CO_2 flow be maintained without interruption throughout each assay. All assays are done twice, and the reaction times averaged.

Blank Reaction Time (Tuc)

This represents the time required for the spontaneous return of the test solution to acidity due to the uncatalyzed hydration of CO_2 when 100 μl of distilled water in .05%

triton is added instead of enzyme prior to the addition of barbital buffer. Tuc typically was 32 seconds.

Catalyzed Reaction Time (Tc)

This is the time required for the color change when 100 μl of 1:100 heparinized whole blood is added prior to barbital. The concentration of enzyme units (EU) is calculated from

$$\text{EU (U/mg Hb)} = \frac{\text{Tuc} - \text{Tc}}{\text{Tc}} \times \text{Dilution} \times \frac{1}{\text{Hb conc.}} \text{(mg/ml)},$$

where Tuc is the uncatalyzed time, and Tc, the catalyzed time in seconds. One unit of catalytic activity of the enzyme speeds the reaction twofold.

Assay in the Presence of Sodium Iodide

Two hundred forty microliters 125 mM sodium iodide is added to 3.760 ml of phenol red. Blank reaction times and catalyzed reaction times in the presence of inhibitors are determined exactly as described above.

Assays following Incubation with Bromopyruvic Acid

One milliliter of the 1:100 heparinized whole blood is mixed with an equal volume of freshly prepared 40 mM bromopyruvic acid in sodium phosphate buffer, pH 6.8. The sample is vortexed and 250 μl removed immediately for the "0" hr assays, providing 100 μl for each of the duplicate assays. The remaining sample is incubated at 25°C, and aliquots of 250 μl are removed at intervals and stored on ice until assayed. Control samples were obtained by diluting the 1:100 dilution of whole blood with an equal volume of sodium phosphate buffer without bromopyruvic acid and removing 250-μl aliquots from this incubation mixture at intervals after placing the mixture at 25°C.

RESULTS

CA I has been reported to be much more sensitive than CA II to inhibition by halide ions. For iodide, this difference is nearly 100-fold (Ki = 0.3 mM for CA I, and 26 mM for CA II) [18]. One can calculate the theoretical inhibition for CA I as 89%, 95%, 97.3%, 98.5%, and 99.8% for I^- concentrations of 2.5, 5.0, 10, 20, and 200 mM, respectively [18]. For CA II, the fractional inhibitions by iodide at these concentrations are only 9%, 16%, 28%, 44%, and 88.5%. This differential sensitivity suggested that CA II might be measured under conditions where its inhibition is minimal, but the activity of CA I is nearly fully inhibited. To explore the possibility of exploiting this differential sensitivity to iodide in assaying CA II in red cell lysates, we examined the effects of iodide on the CO_2 hydratase activity in hemolysates from a CA II-deficient patient, an obligate heterozygote, and a normal control (fig. 1).

We had previously reported that CA I is the only significant isozyme identifiable by HPLC in erythrocytes of these CA II-deficient patients [11]. Thus, we expected only the iodide-sensitive isozyme in this lysate. The data in figure 1 agree with this prediction. Above 5 mM, the inhibition of the enzyme is virtually complete. The hemolysate from the normal control sample contains both CA I and CA II. Although CA I is present in five to six times greater amounts than CA II in erythrocytes [2], the specific activity of CA II is much

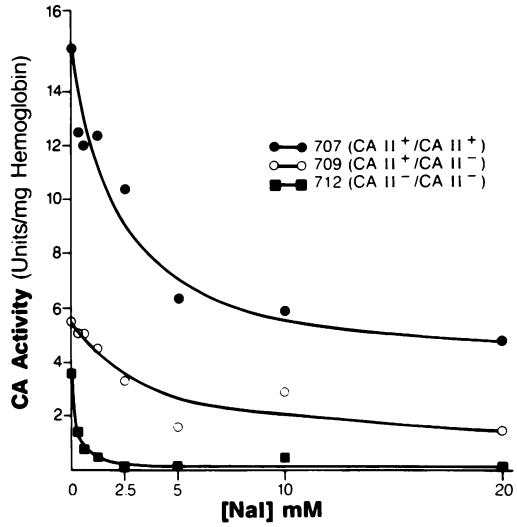

FIG. 1.—Sodium iodide inhibition of human CA I in whole blood lysates from a normal control, an obligate heterozygote, and a patient homozygous for CA II deficiency.

greater, and the contribution of the two to the total CO_2 hydratase activity under these conditions is about equal (actually 52% CA II and 48% CA I). The contribution of two components with different sensitivities to I^- in normal erythrocytes is evident in figure 1. The sensitive component is virtually completely inhibited by 5 mM I^-. The more resistant component, which we assume is CA II, retains substantial activity in 20 mM I^-. The lysate from the obligate heterozygote for CA II deficiency (previously shown by HPLC to have reduced levels of CA II) [11] has a reduced level of total CO_2 hydratase activity with the reduction primarily in the I^- resistant activity.

On the basis of these results, we concluded that one can approximate the CA II level in red cell lysates by measuring the CO_2 hydratase activity in the presence of 5 mM sodium iodide. At this concentration, CA I is 95% inhibited and CA II is only 16% inhibited. Table 1 presents the data derived from application of this assay to lysates of whole blood samples obtained from 22 adult normal volunteer donors. The sodium iodide-resistant activity (9.1 ± 2.7 U/mg Hb) was just below 50% of the mean total activity. Assuming this residual activity is mostly due to CA II and that CA II is 16% inhibited, one would estimate the mean or unhibited CO_2 hydratase activity due to CA II to be 10.3 U/mg Hb. The levels in females in this sample were slightly higher than values for males.

Table 2 presents results from similar assays on lysates of whole blood obtained from two families (see fig. 2) with the syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. Family A (fig. 2) was the family studied in the original report of CA II deficiency in this syndrome [11]. Family B is a recently discovered Italian family on whom the clinical diagnosis of the syndrome in three siblings was clear, but on whom no prior enzyme

TABLE 1

TOTAL AND Na I-RESISTANT CA ACTIVITY IN WHOLE BLOOD LYSATES FROM HEALTHY CONTROL DONORS

	Total activity (U/mg Hb)		Na I-resistant activity (U/mg Hb)	
Controls	Range	Mean	Range	Mean
Males (no. = 11)	13.9–18.9	16.4 ± 3.0	5.0–11.7	8.1 ± 2.0
Females (no. = 11)	14.8–28.5	20.2 ± 4.3	5.4–15.1	9.9 ± 3.0
Total	13.9–28.5	19.1 ± 4.3	5.0–15.1	9.1 ± 2.7

studies had been done. Affected patients in both families have reduced total CA activity (5.1–7.7 U/mg Hb) and 0 to very low sodium iodide-resistant activity. The three heterozygotes previously studied by other means in family A and the two obligate heterozygotes in family B also had reduced levels of total CA activity (10.7–14.4 U/mg Hb) and reduced levels of sodium iodide-resistant activity (4.0–4.8 U/mg Hb). The level of sodium iodide-resistant activity in all five heterozygotes was below 50% of the mean sodium iodide-resistant CA in controls.

Another potential method to discriminate CA I and CA II is based on their differential inactivation by bromopyruvic acid (BPA) [18]. CA I was rapidly inactivated by BPA when incubated with the enzyme at pH 7.6 at 25°C, an

TABLE 2

TOTAL AND Na I-RESISTANT ACTIVITY IN WHOLE BLOOD LYSATES FROM FAMILIES WITH OSTEOPETROSIS WITH RENAL TUBULAR ACIDOSIS AND CEREBRAL CALCIFICATION

Family A	Total activity (U/mg Hb)	Na I-resistant activity (U/mg Hb)
I-1	14.4	4.4
II-2	13.6	4.7
II-3	10.7	4.0
III-1	6.6	0
III-2	5.1	0
Control 1	21.9	10
Control 2	28.5	13.2
Control 3	19.5	9.4
Family B	**Total activity (U/mg Hb)**	**Na I-resistant activity (U/mg Hb)**
I-1	11.7	4.1
I-2	12.7	4.8
II-1	7.7	1.1
II-2	7.2	0.4
II-3	6.0	0
Control 1	17.9	8.7
Control 2	25.2	10.6

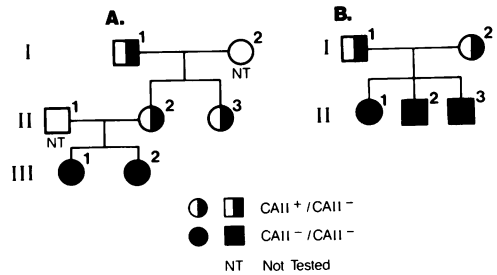

FIG. 2.—Pedigrees of two families with osteopetrosis with renal tubular acidosis and cerebral calcification.

effect postulated to be due to binding to and modification of a histidine residue (residue 200) at or near the active site of the CA I molecule [18]. CA II lacks a histidine residue at this site and was found to be very resistant to inactivation by this reagent, but it was studied at pH 6.8 in phosphate buffer. We chose the latter condition to study the inactivation of the mixture of CA I and CA II in red cell lysates.

Figure 3 shows the effect of incubation with BPA on lysates from a CA II-deficient patient, an adult control, and an obligate heterozygote for CA II deficiency. All of the residual activity in the CA II-deficient patient (previously shown to be principally CA I) [11] was completely inactivated by 90 min at 25°C. A sample from the control subject shows both a BPA-sensitive and a

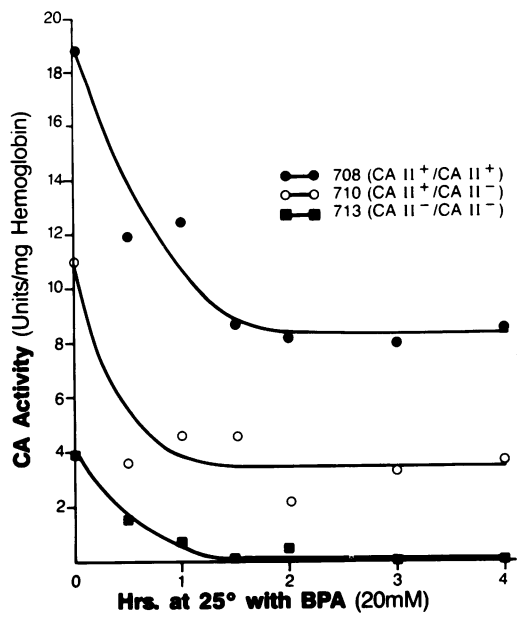

FIG. 3.—Bromopyruvate inactivation of human CA I in whole blood from a normal control, an obligate heterozygote, and a patient homozygous for CA II deficiency.

BPA-resistant component. The obligate heterozygote showed a somewhat reduced BPA-sensitive fraction and a significantly decreased BPA-resistant (CA II-like) activity.

The assay was then used to collect data from the 22 healthy adult donors used in the studies discussed above. Total CA activity was 20.9 ± 3.6 U/mg Hb, and the mean BPA-resistant activity (presumably CA II), 10.7 ± 2.5 U/mg Hb (table 3). Females again scored slightly higher than males in both the total and BPA-resistant CA activity. As was noted for the sodium iodide-resistant CA activity, the BPA-resistant activity was approximately 51% of the mean total activity. In table 4 we present data from similar assays using bromopyruvic acid on whole blood obtained from families A and B (fig. 2). In both families, members affected by osteopetrosis with renal tubular acidosis and cerebral calcification demonstrated significantly reduced CA activity (mean 5.8 U/mg Hb) and zero levels of BPA-resistant activity. Heterozygotes had a mean total activity of 11.7 U/mg Hb and a mean BPA-resistant activity of 4.0 U/mg Hb.

DISCUSSION

The evidence from prior studies [14, 15] indicates that CA II deficiency is the enzymatic basis for the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. To date, there has been no exception to the finding of profound CA II deficiency in erythrocytes of patients with this syndrome (21 patients from 13 unrelated families). In all but one pedigree, reduced levels of CA II could be demonstrated in red cell lysates of parents of these patients by the HPLC method. However, success with this method required careful standardization of the HPLC column with multiple control samples each time the assay was attempted and careful control of the conditions of shipment of blood and interval between collection and processing. Assay of fresh (unfrozen) hemolysate had to be done immediately after the hemolysate was prepared. These stringent limitations precluded verification of carrier status by this method in one family from Saudi Arabia, although diagnosis of the affected members of this pedigree by HPLC posed no problems.

The results presented in this report suggest two alternative methods of docu-

TABLE 3

TOTAL AND BPA-RESISTANT CA ACTIVITY IN WHOLE BLOOD LYSATES FROM HEALTHY CONTROL DONORS

	TOTAL ACTIVITY (U/mg Hb)		BPA-RESISTANT ACTIVITY (U/mg Hb)	
CONTROLS	Range	Mean	Range	Mean
Males (no. = 11)	15.8–26.7	20.4 ± 3.3	6.3–14.1	10.0 ± 2.2
Females (no. = 11) ...	16.6–28.1	21.5 ± 4.1	7.8–17.0	11.4 ± 2.6
Total	15.8–28.1	20.9 ± 3.6	6.3–17.0	10.7 ± 2.5

TABLE 4

TOTAL AND BPA-RESISTANT CA ACTIVITY IN WHOLE BLOOD LYSATES FROM FAMILIES WITH OSTEOPETROSIS WITH RENAL TUBULAR ACIDOSIS AND CEREBRAL CALCIFICATION

Family A	Total activity (U/mg Hb)	BPA-resistant activity (U/mg Hb)
I-1	13.2	3.8
II-2	12.7	4.0
II-3	8.8	3.1
III-1	5.8	0
III-2	4.3	0
Control 1	20.7	7.8
Control 2	28.1	13.2
Control 3	16.6	9.7
Family B	**Total activity (U/mg Hb)**	**BPA-resistant activity (U/mg Hb)**
I-1	11.6	4.5
I-2	12.2	3.5
II-1	6.1	0
II-2	6.2	0
II-3	6.7	0
Control 1	22.3	11.3
Control 2	25.6	13.7

menting the deficiency of CA II in erythrocytes of affected patients and of identifying carriers for CA II deficiency. Both assays measure CO_2 hydratase activity in red cell lysates and depend on the selective inhibition (by sodium iodide) or selective inactivation (by bromopyruvic acid) of the CA I enzyme. The data suggest that both methods allow one to measure the CO_2 hydratase activity attributable exclusively to CA II. Since the two assays depend on different principles of measuring CA II in the absence of CA I activity, and they give remarkably similar values for CA II activity, each tends to validate the accuracy of the other. Mean values for total activity were 19.1 ± 4.3 U/mg Hb for the assay on the control samples for sodium iodide-resistant activity and 20.9 ± 3.6 U/mg Hb for the assay on the control samples for bromopyruvic acid inactivation. Sodium iodide-resistant activity was 9.1 ± 2.7 U/mg Hb for the 22 adult controls. Correction of this for the 16% inhibition of CA II by 5 mM iodide and the 5% residual CA I gives an estimate of CA II activity of 10.3. The bromopyruvic acid-resistant activity for the same samples was 10.7 ± 2.5 U/mg Hb. The agreement between the two estimates of CO_2 hydratase activity due to CA II is striking.

The diagnosis of CA II deficiency was unequivocal in the five samples from affected patients that were analyzed by both methods. Sodium iodide-resistant activity and BPA-resistant activity in lysates from affected patients were comparably low. Heterozygote carriers were also scored similarly by the two techniques giving CA II values of 4.0–4.8 U/mg Hb with the sodium iodide inhibition assay and 3.1–4.5 in the BPA inactivation assay.

Not only do these two methods offer advantages in terms of simplicity for the assay (no HPLC apparatus is required), they permit analysis on samples that could not be analyzed with the HPLC assay because of lysis of the blood in shipment. The samples from family B provided a good illustration of this point, as the hemolysis of these samples was near total on arrival (we suspect that they had frozen and thawed during shipment). Consequently, no separation of erythrocytes from serum was possible, and HPLC analysis of these samples could not be carried out. The data shown in tables 2 and 4 indicate that the alternative assay methods described here provided clear-cut diagnoses when the lysed whole blood samples from these patients and the accompanying controls were diluted and analyzed.

There is one additional advantage to the two methods presented. Both of them would detect CA II deficiency in patients who have a catalytic defect in the enzyme, but normal levels of catalytically inactive CA II protein. Since the HPLC determination quantitates the CA I and CA II protein peaks, not the catalytic activities, a patient whose mutation inactivates the CA II catalytic activity but does not reduce the amount of CA II protein in the erythrocyte might be scored as normal by the HPLC method.

An unexpected finding in these studies was the apparent reduction in the CO_2 hydratase activity attributable to CA I in lysates from patients with CA II deficiency and in obligate heterozygote carriers of this mutation. The CO_2 hydratase activity attributable to CA I is the component of the total CO_2 hydratase activity that is sensitive to inactivation by BPA and to inhibition by sodium iodide. In lysates from control subjects, this component averaged about 10.2 U/mg Hb using the BPA inactivation assay and 10.0 on the iodide inhibition assay. However, the residual CO_2 hydratase activity in samples from CA II-deficient patients (all of which is CA I) averaged only 60% of this value (mean = 6.3 U/mg Hb). This result suggested that these CA II-deficient patients also have reduced levels of CA I in their erythrocytes. Since these patients have a metabolic acidosis, and other problems, this could be an indirect consequence of their CA II deficiency mutation. However, the heterozygotes who have no acidosis or other clinical abnormalities, also have a reduced level of CA I in their erythrocytes. The mean BPA-sensitive activity in the five heterozygotes studied here was 7.9 U/mg Hb (i.e., only 80% of the control CA I activity). This result suggests that mutation producing CA II deficiency in these two families may also reduce the expression of the CA I allele. There is evidence, at least in the mouse, that the CA II and CA I alleles are quite closely linked [23]. If this close linkage also exists in humans, the CA II mutation in these families might have a position effect on expression of the CA I gene. An analogous effect of a CA I mutation on expression of the CA II gene has been reported in the pig-tailed macaque, and it was suggested that this CA I mutation may exert a polar effect on CA II synthesis [24, 25].

The CA II gene has been mapped to chromosome 8 [26], the gene cloned [27], and a CA II-linked polymorphism has been identified in humans [28]. However, nothing is yet known of the nature of the molecular genetic defect in any of the patients with the CA II deficiency syndrome. All patients described so far have

a nearly total absence of CA II in erythrocytes (catalytically, immunologically, and by analysis of CA II protein by HPLC). However, the clinical manifestations in the different pedigrees studied thus far vary in severity, suggesting genetic heterogeneity among the CA II-deficient families. Since CA II is expressed in erythrocytes, the diagnosis of the disorder can be made from samples of peripheral blood. The method presented here greatly simplifies the quantitation of CA II in erythrocyte lysates and should make genetic diagnosis and counseling for this new inborn error of metabolism more generally available.

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