Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification

(erythrocytes/bone resorption/parathyroid hormone/isozymes/heterozygote detection)

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ABSTRACT The clinical, radiological, and pathological findings in three siblings affected with the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification have been reported. In an effort to explain the pleiotropic effects of the mutation producing this disorder, we postulated a defect in carbonic anhydrase II (CA II), the only one of the three soluble isozymes of carbonic anhydrase that is known to be synthesized in kidney and brain. We report here biochemical and immunological evidence for the virtual absence of CA II in erythrocytes of patients affected with this condition, whereas CA I level is not reduced. Levels of CA II in erythrocyte hemolysates from asymptomatic obligate heterozygotes are about half of normal. These findings: (i) elucidate the basic defect in one form of inherited osteopetrosis; (ii) provide genetic evidence implicating CA II in osteoclast function and bone resorption; (iii) explain previous observations that carbonic anhydrase inhibitors block the normal parathyroid hormone-induced release of calcium from bone; (iv) clarify the role of renal CA II in urinary acidification and bicarbonate reabsorption; and (v) suggest a method to identify heterozygous carriers for the gene for this recessively inherited syndrome.

Osteopetrosis is an inherited metabolic bone disease in which a generalized accumulation of bone mass prevents normal development of marrow cavities and the enlargement of osseous foramina (1–3). It has been called "marble bone disease" because the bones are very dense radiographically, although the bones typically have an increased susceptibility to fracture (2, 3). Multiple genetic defects produce osteopetrosis but the mechanism common to all the known forms of osteopetrosis is a failure of bone resorption (4). In man, two principal types of osteopetrosis have been described. One is a dominantly inherited, relatively benign condition which is often detected radiologically in asymptomatic adults (2, 3). A second type is the recessive, lethal, malignant form of osteopetrosis. In this form, osteopetrosis is usually present at birth, becomes symptomatic early in infancy, and leads to death in infancy or early childhood from infection or bleeding (3). Forms of osteopetrosis with clinical courses of intermediate severity have also been recognized (3). Various animal models of osteopetrosis have been identified including four different mutations that produce osteopetrosis in mice (4).

In 1972, three separate reports described a distinct form of osteopetrosis that occurred in association with renal tubular acidosis (5–7). The pattern of inheritance was autosomal recessive.

The clinical course was not entirely benign, but the disease was compatible with long survival and the hematologic abnormalities that dominate the clinical picture in the recessive lethal form of osteopetrosis were absent. One of these families was not described in a complete report until 1980 (8), by which time two of the three affected siblings had been found to have calcification of the basal ganglia. In the same year, Ohlsson et al. (9) independently reported three Saudi Arabian families involving first-cousin marriages that produced offspring with osteopetrosis, renal tubular acidosis, and cerebral calcification, a syndrome for which they proposed the name "marble brain disease."

In an effort to explain the pleiotropic effects of the mutation underlying this disorder by a single enzyme defect, we postulated a defect in one of the three isozymes of carbonic anhydrase (CA I, CA II, CA III) which are known to be under separate genetic control in humans (10–14). This hypothesis seemed attractive for two reasons: (i) metabolic acidosis can be produced by sulfonamide inhibitors of CA (12), and (ii) several reports have shown that CA inhibitors can block the parathyroid hormone-induced release of calcium from bone, suggesting a role for CA in bone resorption (15–17).

The relationship of CA deficiency to cerebral calcification was less apparent, although it is known that CA II is present in brain (19) and that CA inhibitors inhibit cerebral spinal fluid production (19) and affect electrical activity of the brain (20). A defect in the CA II isozyme seemed most likely because this is the most widely distributed of the three known soluble isozymes of CA in human tissues (10, 11) and CA II is the only soluble isozyme so far identified in renal and brain tissue (18, 21, 22). In addition, a genetically determined, virtually complete absence of CA I in mature erythrocytes has been found to have no clinical consequences (23). Because both CA I and CA II are expressed in human erythrocytes, it was possible to test this hypothesis by examining these isozymes in hemolysates of peripheral blood from the family we reported previously (8).

In this report, we describe studies showing what appears to be an almost complete absence of CA II in erythrocytes of patients affected with the syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification. Furthermore, we report that in asymptomatic normal parents of affected patients the levels of CA II are half of normal, which supports the interpretation that the CA II deficiency is the basic defect underlying this clinical disorder and suggests a means to identify heterozygote carriers.

Abbreviations: CA, carbonic anhydrase; FTH, parathyroid hormone.
MATERIALS AND METHODS
Preparation of Erythrocyte Hemolysates. Whole blood (about 10 ml) was collected in heparinized tubes (lithium heparin) and shipped by overnight carrier in ice. Immediately on arrival, the erythrocytes were washed three times in 2 vol of 0.85% NaCl at room temperature. Hemolysates were prepared by lysing the cells with 1 vol of distilled water and extracting with 0.4 vol of toluene. This mixture was shaken on a Vortex mixer for 1 min and then centrifuged at 2,250 × g for 20 min. After aspiration of the toluene layer and membrane interface, the aqueous layer was centrifuged at 12,000 × g for 5 min to remove remaining cellular debris.

Electrophoresis, Staining, and Immunodiffusion. Vertical starch gel electrophoresis of the hemolysates was carried out (8 V/cm for 18 hr) with a borate buffer system at pH 8.6 (24). The esterase activities of the electrophoretically separated CA I and CA II isozymes were detected with a mixture of 4-methylumbelliferyl acetate for CA I and fluorescein diacetate for CA II (25); the electrophoretograms were stained with 0.4% nigrosin to detect protein (24).

The antisera to the CA isozymes were prepared as described (26) by injecting rabbits with human CA I and CA II purified from hemolysates by affinity chromatography on sulfonamide-Sepharose and CM-Sephadex columns (27). Double immunodiffusion on agar plates (1.5% agar in 0.2 M sodium citrate-buffered saline, pH 6.7) was carried out by standard methods.

Quantitation of CA I and CA II. The ratio of CA I and CA II in individual hemolysates was determined by measuring the amounts of CA I and CA II after their separation by reverse-phase HPLC as described (28). Two hundred microliters of fresh hemolysate was extracted with 200 μl of 40% ethanol (kept at −20°C) and 100 μl chloroform (kept at −20°C) by vortexing in a polypropylene Microfuge tube for 1 min at 4°C. A longer extraction time results in some loss of CA II (and a smaller loss of CA I); shorter extraction can result in a pink aqueous layer due to retention of some hemoglobin components. Samples were centrifuged at 12,000 × g for 2 min and the aqueous layer was collected. Fifty-microliter samples were injected into an Altex-Beckman HPLC apparatus containing a Waters μBondapak C18 column. Buffer A (0.1% trifluoroacetic acid [Fierce] in HPLC-grade water [Burridck and Jackson, Muskegon, MI]) and buffer B (0.05% trifluoroacetic acid in UV-grade acetonitrile [Burridck and Jackson]) were used to develop, over 40 min, a 38–46% linear gradient of buffer B. The peaks were monitored at 215 nm, and those corresponding to CA I and CA II were integrated by using a Hewlett-Packard 3390A integrator. The CA I/CA II ratios were calculated without any correction for the small (<10%) differences in extinction coefficients at 215 nm.

RESULTS
Fig. 1 presents an abbreviated pedigree of the family with the recessive syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification that we reported (8). It includes the three affected sisters, their unaffected sister, and the parents who are obligate heterozygotes. Hemolysates from these individuals were used for the electrophoretic, immunologic, and HPLC studies of the CA I and CA II isozymes that are presented below.

The soluble isozymes of CA in erythrocyte hemolysates have high affinities for certain esters and can be assayed on the basis of their esterase activity (24, 25). Fig. 2 presents the patterns of electrophoretically separated CA I and CA II isozymes on starch gels stained for esterase activity and for protein. No CA II isozyme was detected by either stain in the hemolysates of the three affected sisters. The two obligate heterozygotes (II-14, II-15) and the unaffected sister (III-3) showed lower levels of protein and esterase staining than did the controls. These results indicate virtual absence of both CA II esterase activity and stainable CA II protein in the hemolysates of affected patients and decreased levels in the heterozygotes. CA I levels from the same hemolysates were not reduced. In fact, CA I levels appeared to be slightly greater in the patterns of the three affected sisters, suggesting the possibility of a small compensatory increase in CA I in CA II-deficient patients (note that levels of HbA remained essentially the same).

Fig. 3 presents results of double immunodiffusion studies on this family with specific antisera to CA I and CA II. With antisera to CA I, immunodiffusion patterns were normal for all individuals. By contrast, with antisera to CA II there was no crossreacting material in hemolysates from the three affected individuals (III-1, III-3, and III-6). Immunoprecipitin bands in hemolysates from the two obligate heterozygotes (II-14 and II-15) and their clinically unaffected daughter were not different from those of controls (the spouses of III-3 and III-6). Thus, the results of the immunodiffusion studies were consistent with the data in Fig. 2 showing the virtual absence of CA II in the affected sisters but did not detect quantitative differences between heterozygotes and controls.

We next attempted quantitation of the CA I and CA II isozyme levels in individual hemolysates by measuring the amounts of each isozyme after their separation by reverse-phase HPLC. Fig. 4 presents the elution patterns for hemolysates from one of the three affected sisters (III-6), her husband, and the obligate heterozygote mother (II-14) and father (II-15). The CA I/CA II ratios for these individuals were >1.0, 9.0, 16.8, and 16.3, respectively. The virtual absence of CA II in the affected patient was confirmed by this method. Because the higher-than-control ratios in the obligate heterozygote parents suggested that CA I/CA II ratios determined by this method might be

FIG. 1. Abbreviated pedigree of a family with osteopetrosis, renal tubular acidosis, and cerebral calcification. Solid symbols indicate affected sisters.

FIG. 2. Patterns of erythrocyte CA I and CA II of individuals shown in Fig. 1 and of controls, stained for esterase (A) and protein (B) after starch gel electrophoresis for 18 hr in 20 mM sodium borate pH 8.6 buffer at 4°C. Lanes: 1, unaffected control (spouse of III-3); 2, unaffected father (II-15); 3, unaffected mother (II-14); 4, affected proband (III-1); 5, unaffected sister (III-3); 6, affected sister (III-5); 7, affected sister (III-6); 8, unaffected control (spouse of III-6). See Figs. 1 and 5 for individual pedigree designations.
used to determine heterozygosity for the gene for this condition; blood was obtained from additional members of this family and CA I/CA II ratios were determined by HPLC. The ratios fell into three groups: $\geq 10^6$ for affected patients; 6.2-9.8 for one group which included four controls and five unaffected members at risk for heterozygosity; and 13.4-17.2 for a group which included two obligate heterozygotes and five unaffected people at risk for heterozygosity (Fig. 5).

The mean value for the CA I/CA II ratio for controls obtained by using HPLC in this study is somewhat higher than that obtained by radioimmunoassay in a prior study [mean ± SEM: 6.32 ± 0.94 for 58 white control subjects and 6.64 ± 1.37 for 79 black control subjects (24)]. This higher value may reflect the preferential denaturation of CA II by the chloroform/ethanol extraction used to prepare hemolysates for the HPLC analysis. However, the absence of overlap in CA I and CA II ratios determined by HPLC between the small number of controls and the presumed heterozygotes in this study suggests that the CA I/CA II ratio can be used to determine heterozygosity for the gene producing the inherited syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification.

**DISCUSSION**

All three soluble isozymes of CA in humans, CA I, CA II, and CA III, are monomeric, =29,000-dalton, zinc metalloenzymes which catalyze the reversible hydration of CO$_2$ (reaction I) (10–

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+.
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The direction of these reactions depends on the relative concentrations of CO$_2$ and HCO$_3^-$ and on the pH. There is also a distinctive membrane-bound CA in lung, which may represent a fourth enzyme and tentatively is designated CA IV (29). The
kidney also contains a membrane-bound CA that is an intrinsic component of the brush border of the proximal tubule (30–32). Genetic and structural evidence suggests that at least the soluble isozymes comprise a multilocus enzyme family derived from a common ancestral gene by gene duplications (11). However, the kinetic parameters of these isozymes and their sensitivity to inhibitors can differ markedly (33, 34). These findings have suggested that the physiological roles for the different isozymes are diverse, which is also suggested by their different tissue distributions.

The human CA II isozyme, whose turnover number for the CO₂ hydration reaction under physiological conditions (1.3–1.9 × 10⁶ sec⁻¹) is the highest known for any enzyme (35, 36), has been identified (immunologically or by purification) in a wide variety of cells, tissues, and organs including erythrocytes, brain, eye, kidney, cartilage, liver, lung, skeletal muscle, pancreas, gastric mucosa, and anterior pituitary body (10, 11). The other isozymes, whose activities toward HCO₃⁻ and HCO₃⁻ are lower than those of CA II in the order CA II > CA IV > CA I > CA III (29, 33, 34), appear to have a more limited distribution. CA I is found primarily in erythrocytes, CA III mainly in red skeletal muscle, and CA IV in lung.

The finding of a quantitative defect in CA II in these patients provides us with an unusual opportunity to assess the importance and function of this isozyme. In view of the high CO₂ hydrase activity of CA II and its wide tissue distribution, one might expect widespread effects of this deficiency in organs in which CA II plays an important role. However, it should be noted that the finding of a virtual absence of CA II in erythrocytes does not necessarily imply a comparable deficiency in other tissues and organs in which CA II has been reported. CA II levels in cells with considerably more rapid turnover than erythrocytes could be appreciably higher. Also, there may be additional, still unidentified, CA genes contributing to enzyme levels in tissues spared by this mutation. However, what is clear from these patients is that the quantitative deficiency of CA II that we have demonstrated in erythrocytes has important clinical consequences for bone, for kidney, and for brain that merit discussion.

Bone Metabolism. All known forms of osteopetrosis are associated with failure to resorb bone (4). Studies of several patients with osteopetrosis have demonstrated impaired hypercalcemic responses to infused parathyroid hormone (PTH) (37, 38). The cause for this impairment might differ in the different forms of osteopetrosis. Studies showing inhibition of PTH-induced release of calcium from bone by CA inhibitors have suggested a role for CA in bone resorption (15–17). Also, CA has been demonstrated histochemically in chick and hen osteoclasts (39). On the basis of these and other observations (40, 41), it has been suggested that PTH activates CA in certain bone cells where it might aid the resorptive process by mediating secretion of H⁺ (16, 39). The genetic evidence presented here provides strong support for a role of CA in bone resorption, which was suspected from the pharmacological and histochemical evidence cited above, and specifically implicates the CA II isozyme in bone resorption.

Renal Tubular Acidosis. There is general agreement that renal reabsorption of bicarbonate is a major factor in the maintenance of acid-base homeostasis (42). Most of the bicarbonate reclamation takes place in the proximal tubule and depends on CA (43). Only recently has it become clear how both a soluble (cytosolic) and a membrane-bound (luminal) CA play separate roles in the proximal tubule (21, 42–46). Bicarbonate reclamation depends on H⁺ secretion, the major role of CA II in proximal tubular acidification (42). The H⁺ secreted into the lumen of the proximal tubule is titrated by the HCO₃⁻ in the glomerular filtrate to produce H₂CO₃ which is in contact with the membrane-bound CA. The luminal CA then catalyzes the dehydration of H₂CO₃ to CO₂ and H₂O (42, 43). The CO₂ diffuses freely into the proximal tubular cell, where it can be hydrated by the cytosolic CA to H₂CO₃. Dissociation of this product into H⁺ and HCO₃⁻ allows HCO₃⁻ to be transported by unknown mechanisms into interstitial fluid or the peritubular capillary, completing the reclamation of filtered bicarbonate (42, 43). The regenerated H⁺ can be secreted in exchange for Na⁺ to initiate another cycle (42). From the above, it is clear how different CAs operate at different sites to participate in bicarbonate reclamation.

The enzymatic dehydration of H₂CO₃ in the lumen appears to be mediated entirely by the membrane-bound CA present in the brush border of proximal tubular cells (42, 45, 46). Although we have no direct information on the status of this enzyme in the patients described here, we have no reason to suspect that this enzyme, which is biochemically and immunologically distinct from CA II and the other soluble isozymes (21, 22, 31, 32), is defective in these patients. On the other hand, the enzymatic hydration of intracellular CO₂ is presumably mediated entirely by the soluble enzyme CA II, for which these patients are deficient. The renal tubular acidosis present in patients with this syndrome must be explained in this context.

Although the renal tubular acidosis in the different pedigrees has been variable in severity, and somewhat heterogeneous in type, most of the patients reported have a significant proximal defect (47). In those cases in which HCO₃⁻ reabsorption has been adequately studied, a proximal component, evidenced by HCO₃⁻ wasting at normal plasma HCO₃⁻ concentrations, has also been found (47). Thus, the patients appear to have a mixed or hybrid type of renal tubular acidosis which includes both a proximal component and a distal component. The hydration of CO₂ in cells of the proximal tubule presumably is mediated by CA II which appears to be the major (and perhaps only) soluble isozyme present in the kidney (21, 22, 48). Because this reaction generates some of the H⁺ secreted by the proximal tubule, and bicarbonate reclamation in the proximal tubule depends almost entirely on H⁺ secretion (42–44), we can understand why patients with CA II deficiency might have a renal tubular acidosis that includes a proximal component.

The prominent distal component of the renal tubular acidosis in CA II-deficient patients, evidenced by inappropriately high urine pH values when patients were quite acidotic (47), initially was more difficult to understand. A possible explanation was provided recently by immunohistochemical evidence showing much more intense reaction for CA II in the distal tubules (and even in collecting ducts) than in proximal tubules of human kidneys (48). These results suggest that CA II plays a more important role in the distal tubule than was previously suspected (22), either in generating H⁺ or in titrating OH⁻ produced by the proton-translocating ATPase.

The report (23) that a genetically determined, virtually complete, absence of erythrocyte CA I in man is not associated with renal tubular acidosis is consistent with biochemical and immunological evidence that CA II is the only soluble isozyme present in kidney (21, 22, 48). In view of this evidence, and of the findings presented here, it is difficult to understand the significance of the abnormalities in erythrocyte CA I that have been reported in a few patients with distal type renal tubular acidosis (49, 50).

Brain Metabolism. The function of CA II in brain and the reasons for brain calcification in patients with defects in CA II are less well understood. In the central nervous system, CA II is primarily a glial enzyme and occurs predominantly in oli-
gadendrocytes (18). CA II has been identified in brain homogenates, with up to 50% of the activity in a membrane-bound form (51). Although the functions of CA in brain are still speculative, it is worth noting that many of the patients with the syndrome of osteoporosis with renal tubular acidosis and cerebral calcification have significant mental retardation (9). The patients in the family reported here are exceptional in this regard because their IQ scores are in the low normal range (8). Like the mechanism of the cerebral calcification in this syndrome, the mechanism of the mental retardation is not yet clear.

Erythrocyte Function. One might expect some secondary consequences of CA II deficiency in tissues in which the CO2 produced must be delivered to circulating erythrocytes and discharged from the lungs. This transport depends on the ability of the CA activity in erythrocytes to convert metabolic CO2 to HCO3- rapidly in the tissues and to catalyze the reverse reaction in lung capillaries. Although CA II normally accounts for only 14–17% of the CA in erythrocytes (CA I accounts for the rest), it has been estimated that CA II accounts for about 90% of the CA activity of erythrocytes in vitro (36, 52). This estimate is based on the much greater specific activity of CA II compared to CA I and on the much greater sensitivity of CA I to inhibition by the normal chloride concentration of erythrocytes (52). This estimate is in general agreement with the findings by W. R. Chegwidden (personal communication), in a preliminary experiment on the family described here, that the relative CA activities for the HCO3- dehydration reaction in hemolysates from an affected homozygote (III-1), an obligate heterozygote (II-14), and an unrelated control were 0.17, 0.43, and 1.0, respectively, after correction for the nonenzymatic control reaction. Thus, all of the available evidence indicates that CA II is more important than CA I for the CO2 hydrolase reaction in the erythrocyte. However, Wistrand (36) has estimated that only 2% of normal levels of CA activity in erythrocytes would be required for unloading CO2 in lung capillaries at rest, and 4% would be required at work. If all of these estimates are correct, CA I activity alone may be sufficient for this erythrocyte function. The fact that the patients described here have no disability that we can ascribe to the virtual absence of CA II in their erythrocytes supports this conclusion.

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