Annu. Rev. Biochem. 1995. 64:375–401

HUMAN CARBONIC ANHYDRASES AND CARBONIC ANHYDRASE DEFICIENCIES

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KEY WORDS bone resorption, renal tubular acidosis, mental retardation, CO_2/HCO_3^- transport, gene family, isozyme

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ABSTRACT

Carbonic anhydrases (CAs I-VII) are products of a gene family that encodes seven isozymes and several homologous, CA- related proteins. All seven

0066-4154/95/0701-0375$05.00

isozymes have been cloned, sequenced, and mapped, and the intron-exon organization of five genes established. They differ in subcellular localizations, being cytoplasmic (CA I, II, III, and VII), GPI-anchored to plasma membranes of specialized epithelial and endothelial cells (CA IV), in mitochondria (CA V), or in salivary secretions (CA VI). They also differ in kinetic properties, susceptibility to inhibitors, and tissue-specific distribution. Structural and kinetic studies of recombinant natural and mutant CAs have greatly increased our understanding of the structural requirements for catalysis. Studies of the effects of CA inhibitors over many years have implicated CAs in a variety of physiological processes. Analyses of human and animal CA deficiencies provide unique opportunities to understand the individual contributions of different isozymes to these processes.

SUMMARY AND PERSPECTIVE

Carbonic anhydrases (CA I–CA VII) are the products of a gene family that encodes seven distinct isozymes and several additional CA-related proteins with sequence homology but no CA activity. The seven isozymes differ widely in their kinetics, in susceptibility to different inhibitors, in subcellular localization, and in tissue-specific distribution. They participate in a variety of physiological processes that involve pH regulation, CO_2 and HCO_3^- transport, ion transport, and water and electrolyte balance. Functions that depend on CAs, directly or indirectly, include H^+ secretion, HCO_3^- reabsorption, HCO_3^- secretion, bone resorption, and production of aqueous humor, cerebrospinal fluid, gastric acidity, and pancreatic juice. Metabolic roles include important steps in ureagenesis, gluconeogenesis, and lipogenesis.

The past decade has seen a great renewal of interest in CAs as the genes for each of the family members were cloned, their products expressed in large amounts in bacteria and purified, and their structures modified by site-directed mutagenesis and explored by teams of structural biologists and kineticists. Decades of accumulated hypotheses could be tested directly. In addition, many interesting new questions were raised by the discovery of new members of the family.

Adding greatly to interest in this enzyme family was the discovery 11 years ago that CA II deficiency is the basic defect underlying the inherited human syndrome of osteopetrosis with renal tubular acidosis and brain calcification. Studies of patients with this disorder clarified the role of CA II in bone resorption and in renal acidification, and suggested its importance in normal brain development. In addition, these studies illuminated roles for other CAs that were not affected by the mutations producing CA II deficiency. Not surprisingly, the extraordinary lessons provided from this one example stim-

ulated interest in identifying other human CA deficiencies, and in producing animal models for these deficiencies.

References 1–14 provide a partial list of reviews and books on CA research from the last decade. Future research is likely to focus on finding hereditary disorders resulting from deficiencies in the other CAs, developing murine models for each of these enzyme deficiencies using gene-targeted inactivation of the respective gene, defining the regulatory elements that govern the differences in tissue-specific expression of the different isozymes, and defining structure/function correlations that explain their different catalytic properties and susceptibilities to inhibitors. Hopefully, these studies will suggest specific inhibitors for individual isozymes that can be exploited in clinical medicine.

THE CARBONIC ANHYDRASE ISOZYMES—A BRIEF FAMILY HISTORY

Discovery of the first enzyme with CA activity in bovine erythrocytes took place only 60 years ago (15, 16), and recognition of more than one CA in human erythrocytes only 30 years ago (20); characterization of the last four members of the enzyme family took place only in the last five years.

CA activity was studied extensively in various tissues by physiologists and pharmacologists for nearly three decades following its discovery (reviewed in 17, 18) before a CA was first purified in 1960. The enzyme source was bovine erythrocytes, which contain a single, high-activity enzyme (19). Purification from human erythrocytes in 1961 (20) disclosed two forms, one in larger amounts than the other and having less activity, and a second form, present in smaller amounts, that has high activity similar to the bovine enzyme. These high and low activity isozymes, initially designated CA C and CA B, came to be known as CA II and CA I. The amino acid sequences of both CA I (21, 22) and CA II (23, 24) were reported in the early 1970s, and the x-ray crystal structures for CA II (25) and CA I (26) in 1972 and 1975, respectively (27).

Subsequently discovered isozymes were assigned numerical names in the order of their discovery. A sulfonamide-resistant CA was found in homogenates of male but not female rat livers in 1974 (28, 29), and a sulfonamide-resistant CA was isolated from chick muscle two years later (30). Subsequently, it became clear that the same 29-kDa protein had been purified from rabbit skeletal muscle in 1972 (31) and named BMP for basic muscle protein. Its lack of reactivity to antisera to CA I and II, and its insensitivity to sulfonamides, led it to be recognized as a distinct isozyme called CA III (32).

Although membrane-associated enzyme had been reported earlier, its purification from bovine lung in 1982 allowed CA IV to be identified as a distinct isozyme. The lung enzyme required strong detergent to solubilize it from membranes, was 52 kDa, much larger than the 29-kDa isozymes previously

described, was resistant to solubilization in 5% sodium dodecylsulfate, and contained carbohydrate. The human enzyme was isolated subsequently from brush border membranes of kidney (34, 35) and human lung (35) and has been studied intensively over the past five years.

CA V is a nuclear-encoded, mitochondrial isozyme. A CA activity in mitochondria was suspected since 1959 (36) and supported by observed effects of CA inhibitors on mitochondrial metabolism (39). It was demonstrated by O^{18} exchange studies by 1980 (37, 38). Recently, a partial N-terminal sequence was obtained from enzyme isolated from guinea pig mitochondria (40), and a cDNA was isolated from mouse liver that was homologous with the partial sequence from guinea pig and appeared to encode a CA with a mitochondrial leader peptide (41). Expression of this cDNA (42) and the homologous human cDNA (43) allowed the mitochondrial isozyme to be characterized.

Isozyme CA VI is a secretory glycoprotein found in saliva. Although CA activity in saliva was recognized in 1946, it was not until 1979 that salivary CA from sheep was isolated and found to be distinct from other CAs (44). The rat enzyme was characterized in 1984 (45), the human enzyme in 1987 (46), and the complete sequence for the sheep enzyme reported in 1989 (47).

CA VII is unique in that, to this date, it is only a "virtual enzyme." Unlike the other isozymes that were recognized first as proteins for which genes were subsequently identified, the gene for CA VII was identified by homology to other CAs before the protein was recognized (48).

STRUCTURE OF THE CATALYTIC SITE AND REACTION MECHANISM

Structure of the Catalytic Site

X-ray crystallographic structures are available for three of the CA isozymes (49). The structure of CA II, the high activity form from human erythrocytes, has been determined and refined to 1.54Å resolution (50–52). The enzyme has a roughly spherical structure with the active site comprising a conical cleft about 15Å deep. One side of the cavity is formed by hydrophobic residues (Figure 1). The other side contains hydrophilic residues including Thr199 and Glu106. The zinc ion is located at the bottom of this cleft, and tetrahedrally coordinated to the imidazoles of the three histidine residues (His94, His96, His119) and to a water molecule (called the "zinc water") that ionizes to a hydroxide ion with a pK about 7 (53). This zinc coordination polyhedron is a conserved feature among CAs.

Amino acid residues Thr199-Glu106 contribute to a hydrogen bonding network with the $Zn\text{-}OH^-$ that maintains the catalytically competent structure optimal for nucleophilic attack by the zinc-bound hydroxide on the CO_2 sub-

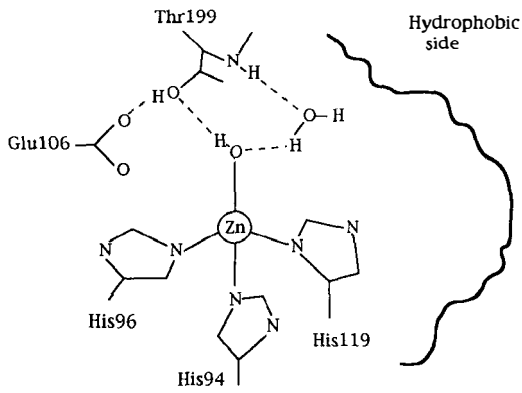

Figure 1 Diagrammatic representation of the active site of CA II. The zinc ion is tetrahedrally coordinated to the imidazole groups of three His residues and to a hydroxide or water (the zinc water) molecule. Thr199 hydrogen binds to the hydroxide ion or water and to Glu106 through its Oγ1. Its peptide nitrogen binds to the "deep water" and may be the site of CO_2 binding (adapted from 10 and 86).

strate (54–59). Thr199 forms two hydrogen bonds from Oγ1, donating a hydrogen bond to the carboxyl group of Glu106 and accepting a hydrogen bond from the zinc hydroxide. The peptide nitrogen of Thr199 also forms a hydrogen bond to a second water molecule in the deepest end of the cavity (referred to as the "deep water"). The Zn-OH^-/Thr199/Glu106 hydrogen bond network, which is absolutely conserved in all nonplant CA isozymes (4, 8), restricts the orientation of the zinc-bound OH^-, so that one of its lone electron pairs is directed toward the CO_2 molecule located in a hydrophobic pocket (55, 58).

Structural studies of inhibitors (10, 60–70) and HCO_3^- (71) binding to the metal ion show that the Zn-OH^-/Thr199/Glu106 network is also important for binding bicarbonate, sulfonamide inhibitors, and many anionic inhibitors (see Ref. 10 for a comprehensive review of these studies). Almost all inhibitors appear to displace the deep water situated between the NH of Thr199, the zinc ion, and the hydrophobic portion of the active site (Figure 1). Some coordinate with the metal ion, and many contribute a hydrogen bond to the hydroxyl of Thr199. In fact, the position of the Thr199 and its ability to offer a lone pair of electrons and act as a hydrogen bond acceptor has led to the suggestion (10, 52, 59) that Thr199 plays a gatekeeper function, limiting access of the metal ion to anions and inhibitors that can serve as hydrogen bond donors. However, some inhibitors like azide can bind the metal ion without being able to contribute a hydrogen bond (66). In this case, the zinc-bound azide nitrogen makes a nonhydrogen bonded van der Waals contact with the hydroxyl group of Thr199 (66). Cyanide and cyanate displace the deep water and bind in the hydrophobic cavity without binding zinc. Instead, they hydrogen bond to the

NH of Thr199 in the binding site, which was suggested to be the position of the substrate CO_2 when attacked by the zinc bound hydroxide (57, 71).

The Catalytic Mechanism

The CAs efficiently catalyze the hydration of CO_2 to bicarbonate and a proton. One isozyme, CA II, is one of the fastest enzymes known, with a maximum turnover rate for the CO_2 hydration reaction of more than 10^6 s^{-1}. CAs also catalyze the hydrolysis of aromatic and aliphatic esters. The kinetics of enzyme catalysis and inhibition have been studied extensively and recently reviewed (9, 53, 72). Considerable evidence suggests that the reaction involves two steps (53, 57), conversion of CO_2 to HCO_3^-, leaving water as a ligand on the zinc (equation 1), and transfer of proton to solvent buffer through a proton shuttle group, His64 (equation 2).

$$\text{EZn-OH}^- + \text{CO}_2 \leftrightarrow \text{EZn-HCO}_3^- \xrightarrow[\text{B}]{+\text{H}_2\text{O}} \text{EZn-H}_2\text{O} + \text{HCO}_3^- \quad 1.$$

$$\text{His64-EZn-H}_2\text{O} \leftrightarrow \text{H}^+\text{-His64-EZn-OH}^- \xrightarrow[\text{BH}^+]{} \text{His64-EZn-OH}^- \quad 2.$$

In the first step shown in Equation 1, the zinc-bound OH^- adds to CO_2 to yield a bicarbonate with a hydroxyl coordinated to the zinc (Figure 2a). In the second step, displacement of the zinc-bound bicarbonate by a water molecule releases bicarbonate and restores zinc-bound water. The proton release reaction (Equation 2), which is facilitated by His64, is the rate-limiting step for the high activity isozymes like CA II (53, 73) (Figure 2b). Inhibition of CA II by heavy metals like copper and mercury is explained by structural studies showing that they bind to His64 (69). The dependence on buffer to accept the proton shuttled by His64 explains why a minimum buffer concentration is required to achieve the maximal rate of turnover (53, 73, 75). Replacement of His64 in CA II with Ala by site-directed mutagenesis results in dramatic decreases in k_{cat} in the CO_2 hydration reaction, with the actual magnitude of the decreases depending on the pH, the buffer, and the buffer concentration (73–75). In fact, the activity of His64Ala CA II is nearly restored to that of wild type in the presence of sufficient imidazole buffer (and several other buffers) which apparently can bypass the rate-limiting proton shuttle and directly accept protons from the zinc water (73, 74). When additional single mutations were made on the background of the His64Ala mutant enzyme to introduce a histidine at position 62, 67, or 200, the k_{cat} of the enzyme increased to nearly 5% of that of the wild type enzyme (76). These results show, on the one hand, that histidine groups at certain other locations can participate in proton transfer.

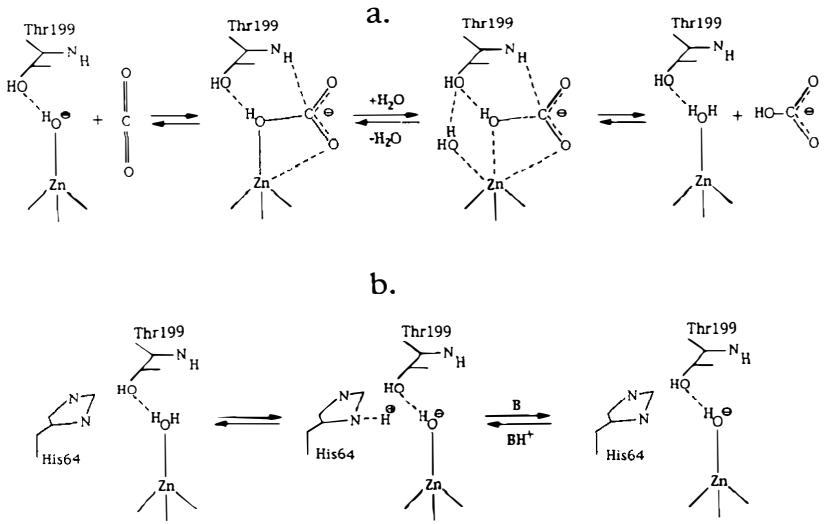

Figure 2 *a.* Diagrammatic representation of CO_2/HCO_3^- interconversion. *b.* Diagrammatic representation of the proton transfer step in which a proton from the zinc water transfers to the His64 proton shuttle from which it transfers to solvent buffer.

On the other hand, they show that the location of the proton shuttle at His64 is critically important for optimum proton transfer.

Other combinations of kinetic and structural studies on wild type and mutant enzymes produced by site-directed mutagenesis have clarified a number of other important structure-function relationships. These include the role of His200 in explaining the higher affinity of CA I than CA II (Thr200) for HCO_3^- (71, 77, 78), the zinc binding coordination and structure of several enzyme-inhibitor complexes (10), the mobility of the protein shuttle residue His64 (79), the key roles of Glu106 and Thr199 in the catalytic function of CA II (57, 58), and the dispensability of Tyr7 for catalytic function of CA II (58). Studies of the hydrophobic pocket adjacent to the zinc-bound hydroxide (80-83) have implicated this region as the site for CO_2 association, provided evidence that this region modulates the catalysis of CO_2 hydration, and defined the structural requirements for the hydrophobic residues in this region.

Differences in properties between the naturally occurring isozymes CA II and CA III have been explored by site-directed mutagenesis. Changing Phe198 in CA III to Leu198 to make it correspond to CA II at this position did increase the k_{cat}/K_m 25-fold and made it more CA II–like (84). However, replacing four active site residues of CA II (His64, Asn67, Leu198, and Val207) with Lys64, Arg67, Phe198, and Ile207, as found in the low-activity muscle-specific CA III, did not reduce the level of activity of the modified CA II nearly to that of

CA III (85). Additional structural features must contribute to the activity differences between CA II and CA III.

As was the case with His64Ala, several individual replacements have been particularly instructive in testing hypotheses regarding structural requirements for catalysis. Among these are the substitutions for Thr199 and Glu106 that alter the hydrogen bonding network of the active site (57–59). Replacement of Thr200 in CA II with His, the residue normally found in this position in CA I–type enzymes (77, 78), also dramatically affects catalysis, in large part by increasing the affinity for HCO_3^- and making HCO_3^- dissociation a rate-contributing if not rate-limiting step for CO_2 hydration by Thr200His CA II, as it is for CA I (77, 78). It was this substitution that stabilized the bicarbonate complex with Thr200His CA II sufficiently to allow structural studies of the enzyme-bicarbonate complex (71). The dramatic progress in this area has been summarized in recent reviews (10, 86).

CONSERVATION OF PRIMARY STRUCTURE AMONG THE HUMAN CAS

Figure 3 (p. 388) presents the alignment of the amino acids in the seven human isozymes, as determined directly or deduced from their cDNAs (13, 87–90). Numbering is based on that of CA I. The 40 residues boxed (15% of the total aligned sequence) are identical in all seven CAs. Comparisons of sequences of each CA with those of the others, and those of CAs from other amniotes, shark, and algae, have been used to deduce the evolutionary patterns of descent from an ancient common precursor (4, 8, 13). It was estimated that CA IV and CA VI are the oldest mammalian CAs evolutionarily, that CA I, II, and III diverged most recently (i.e. between 300 and 400 million years ago), and that CA V and CA VII occupy intermediate positions. The percent identities in the human CAs are consistent with this interpretation. Thus, the putative latecomers, CAs I, II, and III, show 58–60% identity with each other in amino acids at similar positions (46). The percent similarities between the more ancient CA IV and other CAs are: CA I, 31%; CA II, 36%; CA III, 33%; CA VI, 33%; and CA VII, 32% (87). Intermediate is the sequence of CA V, the most recent human CA sequence reported, which reveals the following percent identities with other CAs: CA I, 47%; CA II, 49%; CA III, 44%; CA VII, 48%; CA IV, 30%; and CA VI, 35% (43).

Note that CAs IV, V, and VI have N-terminal and C-terminal sequences that extend beyond the regions of alignment with the cytoplasmic CAs, CA I, II, III, and VII. The N-terminal sequences of CAs IV, V, and VI were deduced from their cDNAs but are not present in the mature proteins. The 18 and 17 amino acid hydrophobic signal peptides at the N-termini of CA IV and CA VI are presumed to be removed cotranslationally as the enzymes are synthe-

sized and translocated into the endoplasmic reticulum of the cells that express them. Likewise, the precursor for CA V contains a 38–amino acid N-terminal mitochondrial leader peptide that is not present in the mature protein isolated from mitochondria (43, 88). The N-terminal leader peptide is assumed to be removed following import into mitochondria.

Both CA IV and CA VI also have additional C-terminal amino acids that extend beyond the aligned sequences. In the case of CA IV, C-terminal processing removes the C-terminal 28 amino acids, and the C-terminus of the 266 amino acid cleaved amino terminal portion of the CA IV precursor is transferred to a glycosylphosphatidylinositol (GPI) anchor (87). Thus, the C-terminal 28 amino acids in CA IV (underlined in Figure 3) do not appear in the mature protein. By contrast, the 30–amino acid C-terminal extension of CA VI is not removed from the secretory glycoprotein, based on the 36–kDa size of the human salivary glycoprotein following removal of carbohydrates (46).

PROPERTIES OF INDIVIDUAL HUMAN ISOZYMES

The Cytosolic Isozymes CA I, II, III, and VII

CARBONIC ANHYDRASE II CA II is a high-activity isozyme with a maximum turnover rate for CO_2 hydration of 1×10^6 s^{-1} (91), and has the widest distribution, being expressed in the cytosol of some cell types in virtually every tissue or organ (13). Cell types expressing CA II include osteoclasts in bone, oligodendrocytes in brain, epithelium of the choroid plexus (brain) and the ciliary body (eye), lens, Müller cells in retina, liver (mainly perivenous hepatocytes), kidney (proximal tubule, distal tubule, and intercalated cells of the cortical collecting ducts), acinar cells in salivary glands, pancreatic duct cells, gastric parietal cells, endometrium of the uterus, endothelial cells, epithelial cells of seminal vesicle and ductus deferens (92), spermatozoa (93), erythrocytes, and platelets. It has also been reported recently in neutrophils (94), type II epithelial cells of lung (95), endothelial cells and epithelial cells of duodenum, intestine, and colon (96), and zona glomerulosa cells of the adrenal (97).

The physiological roles of CA II in these cell types are diverse (6, 13). In some cells, CA II plays a major role in contributing to acid base homeostasis. It contributes to H^+ secretion by gastric parietal cells, by renal tubular cells that secrete H^+ to produce urinary acidification, and by osteoclasts that secrete H^+ to acidify the bone-resorbing compartment. CA II promotes HCO_3^- secretion by pancreatic duct cells that contribute HCO_3^- to pancreatic juice, by ciliary body epithelium (which produces aqueous humor), by choroid plexus (which produces cerebrospinal fluid), by salivary gland acinar cells (which produce saliva), and by distal colonic epithelium, where H^+ and HCO_3^- secretion are coupled to Cl^- and Na^+ reabsorption and contribute to electrolyte and water

balance (98). CA II also promotes CO_2 exchange in proximal tubules in the kidney, in the erythrocytes, and in lung. It has been suggested that it contributes to fatty acid and amino acid synthesis (13).

The transcriptional regulation that appears to account for the different tissue-specific expression of CA II was recently reviewed (13), as was the evidence for hormone regulation of CA II in uterus, bone, and prostate. Recent evidence suggests that there is up-regulation of CA II in kidney in metabolic acidosis (99).

CARBONIC ANHYDRASE I CA I is five to six times as abundant as CA II in human erythrocytes, but has only about 15% of the activity ($k_{cat} = 2 \times 10^5$ s^{-1}) (91). Thus, in intact adult erythrocytes, CA I contributes about 50% of the total CA activity (100). CA I is much more sensitive to inhibition by Cl^- and other halide ions than is CA II, and less sensitive to sulfonamide inhibitors. It is expressed in epithelium of the large intestine, corneal epithelium, lens, ciliary body epithelium, sweat glands, adipose tissue, and myoepithelial cells (13). Recent reports add neutrophils (94) and zona glomerulosa cells of the adrenal gland (97) to this list.

CA I is not detectable in human fetal erythrocytes but is switched on at 40 weeks gestation at the time of expected normal birth (101). It rises to adult levels over the first year of life. Transcriptional and developmental regulation of the CA I gene have been reviewed recently (13). CA I is also strongly expressed in colon, but from a colon-specific promoter that is distinct from the erythrocyte promoter (5, 102, 103).

Although CA I is the most abundant nonhemoglobin protein in red blood cells, no hematologic abnormalities result from its absence (13). In addition, since neither felids nor ruminants express erythrocyte CA I (4), its physiological importance is unclear. Presumably, other CAs or other mechanisms can compensate for its absence in isolated CA I deficiency. However, one might expect CA I to be essential for survival in the absence of CA II.

CARBONIC ANHYDRASE III CA III is a very low-activity isozyme, its activity for CO_2 hydration being only 1% that of CA II. Part of its low activity is attributable to absence of the His64 to carry out the proton shuttle function (73, 104). Part is attributable to the presence of Phe198 in place of Leu198 (as in CA II) (105, 106). Changing Phe198 to Leu198 increases catalytic activity by 25-fold (105). CA III is quite insensitive to inhibition by concentrations of sulfonamides that readily inhibit the other isozymes. Phe198 probably also contributes to sulfonamide resistance by sterically inhibiting access to the cavity (107). Because of its sulfonamide resistance, the CO_2 hydration activity observed in tissues in the presence of 1–5 μm acetazolamide is generally assumed to be CA III (108).

Its major site of expression is skeletal muscle, where it can represent 8% of

the soluble protein of slow-twitch (type I fiber) red skeletal muscle. It is expressed in other fiber types in lower amounts (109) and in high levels in adipose cells, at least in the rat (110). CA III is expressed at lower levels in other tissues, including salivary glands, smooth muscle cells in uterus, red cells, prostate, lung, kidney, colon, and testis (4).

Developmental studies show that CA III is expressed at low levels in human muscle during early fetal development but increases rapidly during the last trimester to reach 50–60% of adult levels at birth (111, 112). Developmental studies have also been done in the rat, mouse, and in myogenic cell lines (reviewed in 13). Of interest is the expression of CA III in notochord at even higher levels than in developing muscle in the embryonic mouse (113).

Although CA III was first purified in 1972, and we have since learned a great deal about its structure, kinetics, hormonal regulation by thyroid (reviewed in 4), and developmental regulation, its functional role is still a mystery (81).

CARBONIC ANHYDRASE VII As mentioned earlier, CA VII is only a virtual enzyme which we know conceptually from its gene, its cDNA, and its mRNA detected by in situ hybridization (13, 48). The mRNA is expressed primarily in the cytosol of the salivary glands. The cDNA has not yet been expressed, nor has the protein been isolated from salivary glands. Thus, its activity, properties, and functional role remain speculative.

The Membrane-Associated CA, CA IV

CARBONIC ANHYDRASE IV CA IV is a fascinating isozyme whose importance has been appreciated only recently. It was originally purified from bovine lung and found to be a membrane-associated glycoprotein of 52 kDa that was resistant to SDS, apparently stabilized by disulfide bonds (33). The purified enzyme was a high-activity enzyme like CA II and was even more resistant to halide ions, although somewhat less resistant to sulfonamide inhibitors. The human enzyme was found to have many similar properties, but it is smaller (35 kDa) and contains no carbohydrate (34, 35). In lacking carbohydrate, the human enzyme is unique among the nine mammalian CA IVs so far studied. The CA IVs vary in molecular mass from 39–52 kDa, and contain 1–5 N-linked oligosaccharide chains. All of the other mammalian CA IVs are reduced to 35–36 kDa when the enzymes are treated with endoglycosidase F to remove N-linked oligosaccharide chains (114).

CA IV, the only membrane-anchored CA, is anchored to membranes by a glycosylphosphatidylinositol anchor and can be released from membranes by treatment with phosphatidylinositol-specific phospholipase C (35, 87, 114).

The cDNA for the human enzyme has been cloned, sequenced, and expressed, and the biosynthesis and turnover of the enzyme studied (87). The genomic sequences have also been characterized (115). Recent kinetic studies (116) confirmed its high-activity character, its resistance to salt inhibition, and its somewhat decreased sensitivity to sulfonamide inhibitors (averaging a 17-fold decrease in sensitivity compared to CA II with a battery of different sulfonamide inhibitors).

CA IV is expressed on the apical surfaces of epithelial cells of some segments of the nephron, the apical plasma membrane in the lower gastrointestinal tract, and the plasma face of endothelial cells of certain capillary beds. In rat kidney, it is highly expressed on the apical brush border membranes (and somewhat less expressed on the basolateral membranes) of proximal tubular cells and cells of the thick ascending limb (117). In the lung, it is expressed on the plasma face of the pulmonary microvasculature (118). Developmental studies show that the CA IV mRNA is not expressed in fetal lung (though it is expressed in fetal kidney). Comparisons of mRNA levels in the adult rat by northern blot analysis suggested that the highest levels of expression are in colon, followed by brain, lung, kidney, and heart. In brain, CA IV has been localized to the plasma face of the cortical capillaries and has been proposed as a marker for the blood-brain barrier (119). CA IV is also expressed on the plasma face of endothelial cells of the choriocapillaris of human eye (120), the microcapillaries of skeletal and cardiac muscle (121), and the microvasculature in rat and human colon (S Parkkila, WS Sly, unpublished observations). It has also been demonstrated on specific epithelial cells of the human reproductive tract (122) and abundantly expressed on the apical plasma membrane of the colon (S Parkkila, WS Sly, unpublished observations).

The physiological role of CA IV in kidney is to facilitate HCO_3^- reabsorption by catalyzing its dehydration to CO_2 (11, 12). In fact, CA IV is the luminal CA in the proximal tubule of the kidney that was inferred from micropuncture studies to mediate 85% of bicarbonate reabsorption in kidney. In other tissues (like brain, skeletal muscle, and heart muscle), it promotes CO_2 flux by facilitating CO_2 hydration to form bicarbonate and accelerating CO_2 removal by the microvasculature from tissues generating CO_2 from metabolism. In lung, it acts to promote CO_2 exchange from the blood to the alveoli by facilitating HCO_3^- dehydration to CO_2 which diffuses across the alveolar membrane. In colon, its role is less clear. Since Na^+ and Cl^- reabsorption are coupled to HCO_3^- secretion that is inhibited by CA inhibitors, a CA is known to be involved (98). The relative contributions of cytoplasmic and membrane CAs to this process are not yet clear. CA IV may facilitate recycling of secreted HCO_3^- and H^+, converting them to CO_2 which can be reabsorbed to promote another round of Na^+ and Cl^- uptake.

The Secretory Form of CA in Saliva, CA VI

CARBONIC ANHYDRASE VI Human CA VI is a 42-kDa glycoprotein that has been purified and characterized from human saliva (46). Its cDNA was cloned from human parotid. The deduced amino acid sequence presented in Figure 3 is 72% identical to that of sheep CA VI (89), and the two cysteines, which were shown to be linked by a disulfide bridge in the sheep enzyme, are conserved. The human cDNA predicts three potential glycosylation sites. Complete and partial digestion products of 36 kDa and 39 kDa, respectively, following treatment with endoglycosidase, suggest that two of these sites are used in the human enzyme (46).

CA VI has also been purified from rat saliva and characterized (45). Both the rat CA VI and the sheep CA VI resemble CA II in being high-activity isozymes (42, 45). The purified human isozyme had considerably lower activity (only 2–3% of that of CA II) (46). Although it resembles CA II in some of its inhibitory properties, it is distinct from CA II in its sensitivity to chloride, acetazolamide, and methazolamide (46).

Human CA VI was found to be expressed only in salivary glands, being prominent in the serous acinar cells of the submandibular gland and parotid gland (124). The mean enzyme content in saliva is 6.8 + 4.3 μg/ml (125), and the estimated daily output in saliva is 10–14 mg per day. Although the cytoplasmic CA II in submandibular and parotid glands mediates HCO_3^- secretion into saliva, the secreted CA VI probably plays a role of pH regulation in saliva, using the buffer provided by CA II, and may have a protective effect in esophagus and stomach (126).

Mitochondrial CA, CA V

CARBONIC ANHYDRASE V The cDNA for human mitochondrial CA V was recently cloned from a human liver cDNA library (43). See Figure 3 for the deduced amino acid sequence. Expression of the cDNA in COS cells produced proteins of the masses expected for the precursor (34 kDa) and mature (30 kDa) mitochondrial enzyme. Only the 30-kDa protein was detected in mitochondria isolated from adult human liver. Processing from precursor to mature CA V in COS cells involves removal of a 38-amino acid mitochondrial leader sequence. Because the amount of activity expressed was only 0.06% of the activity expressed from the same vector containing the cDNA for human CA II, the human CA V was thought to be a "low-activity isozyme." However, Heck et al (42) recently reported purification of mature murine CA V from bacteria expressing the murine cDNA. They found that the k_{cat} for hydration increased dramatically with pH, with an apparent pKa of 9.2. The k_{cat} at pH 7 was only $2.2 \times 10^3\ s^{-1}$, but it increased with pH to $3 \times 10^5\ s^{-1}$ at pHs above 9.2. From these results, they concluded that the activity at alkaline pH for

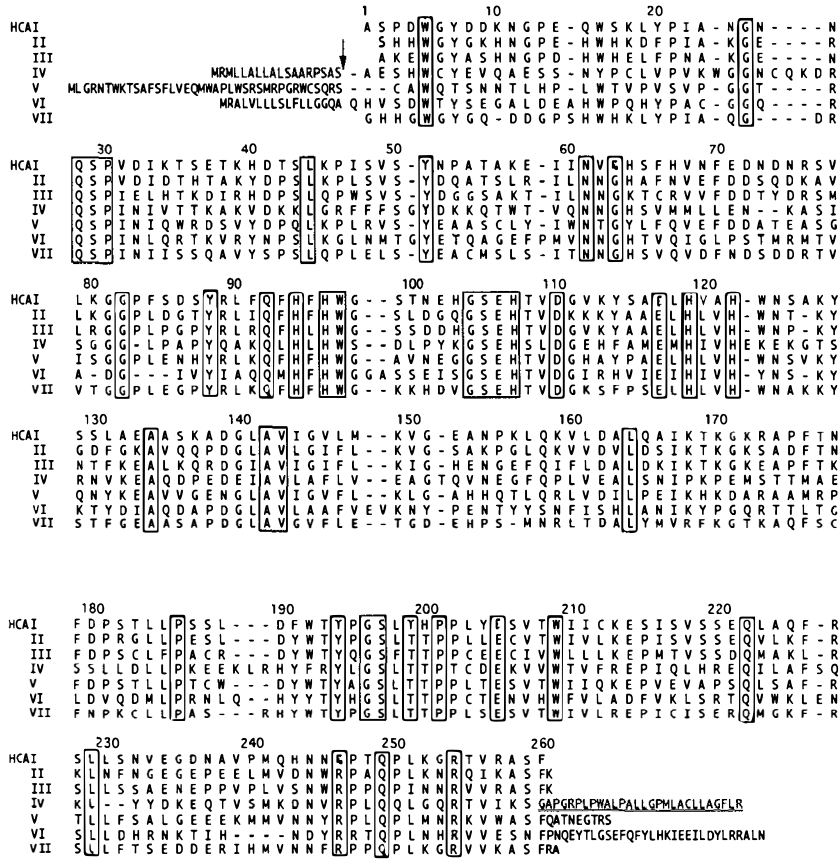

Figure 3 Comparison of amino acid sequences of human CAs I-VII. Homologous residues in all seven isozymes are boxed. Gaps are introduced to optimize alignment. The arrow (upper left) indicates the sites of cleavage of the leader sequences of CA IV, V, and VI. Amino acids to the left of the arrow are deduced from the cDNA sequences and are not found in the mature CA IV, V, or VI. The underlined hydrophobic C-terminal sequence of CA IV is cleaved off during GPI anchoring and not found in the mature GPI-anchored enzyme. The numbering system used is based on that of human CA I. Sequence data for CA I, II, III, and VII are taken from Ref. 13. Sequences for CA IV, V, and VI are from references 87, 88, and 89, respectively.

mouse CA V is quite appreciable. Although the enzyme resembled CA I in its catalytic properties, its sensitivity to inhibitors most resembled those of CA II. Thus, the suggestion that human CA V is a low-activity isozyme, based on the activity expressed in crude extracts of transfected COS cells (43), should be viewed with caution. Also, an earlier estimate of the turnover number of

24,000 s^{-1} for CA V purified from rat liver (127) refers to studies at pH near 7. Possibly, the maximal k_{cat} of the rat enzyme is considerably higher (42).

There are two obvious differences in amino acids near the active site between human CA V and CA II. Tyr7 is replaced in human CA V by Thr, and His64 is replaced by Tyr. Heck et al (42) replaced Tyr64 with His64 in the murine enzyme by site-directed mutagenesis and concluded that the unique kinetic properties of murine CA V are not explained by the Tyr64. The replacement of Tyr7 in CA V with Thr is probably not a critical difference either because Liang et al reported that Tyr7 is not essential for CA II (58).

The distribution of CA V in human tissues has not been established. The mouse mRNA for CA V was detected only in liver on northern blots of mRNA from seven tissues examined (41). Rat CA V has been detected in many more tissues (128, 129), but there is some question whether the antibody used in these studies may have recognized malate dehydrogenase instead of CA V (130). In a recent study using antibodies to synthetic peptides corresponding to the C-termini of mouse and rat CA Vs (131), the mouse antibody detected CA V only in liver of nine tissues examined. However, CA V was detected in six of nine rat tissues (130). The signal was most intense in rat liver, followed by heart, lung, kidney, spleen, and intestine. No signal was detected in brain, testis, or muscle.

CA V has been suggested to be important for two metabolic pathways that depend in part on mitochondrial enzymes (39). One is gluconeogenesis, where CA V may be required to supply HCO_3^- to pyruvate carboxylase in mitochondria. The second is ureagenesis, where CA V may be required to supply HCO_3^- to carbamyl phosphate synthetase in mitochondria. Involvement of CA V in these processes has been suggested to explain the effects of CA inhibitors on these pathways (reviewed in 132).

Other CA-Related Proteins and Unrelated CAs

CA-RELATED PROTEINS There are several examples of proteins with sequence homology to CAs that do not have CA activity. One is a gene product found in vaccinia virus encoded by the D8 gene (133). This intronless gene encodes a 304–amino acid, transmembrane protein with over 30% similarities to mammalian CAs. Its extracellular domain is CA-like, but it has no CA activity. It is a nonessential viral protein, the function of which is unclear. Another viral example is the *erb-A* gene of avian erythroblastosis virus (AEV) (134). The 3′ end of this gene (domain 2) encodes a CA-like domain with similarity to residues 10–183 of CA I. Presumably, both of these viral examples reflect resourceful acquisition of CA sequences for some structural purpose by organisms that did not need to conserve the active site residues required for CA activity.

Three mammalian examples have also been discovered recently. A CA-related mouse brain cDNA was isolated, the deduced sequence of which shows 33–41% homology with CA I, II, III, and VI (135). Two active site substitutions, His94Arg and Gln92Glu, explain its lack of CA activity. This protein, named CARP for CA-related protein, is expressed in Purkinje cells. The human homologue is 98% identical (136), reflecting a high degree of conservation for this Purkinje cell protein with a yet unknown function.

Another highly conserved CA-related protein isolated from a cDNA library from brain is a member of the protein tyrosine phosphatase called PTP-zeta (137). This transmembrane protein has a cytoplasmic domain that encodes two PTPase domains, only one of which is active, and has a large 1616–amino acid extracellular receptor domain whose N-terminal 266 amino acids are homologous to CA. The CA-like structure was proposed to bind a small molecule ligand. The same gene was cloned by another group (138), who mapped it to human chromosome 7 and found its expression limited to brain.

Another CA-related tyrosine phosphatase, RPTP gamma, is encoded by a candidate tumor suppressor gene for renal cell carcinoma that maps to human chromosome 3p14.2-p21 (139, 140). It also has two tandem PTPase domains in the cytoplasmic tail, and its large extracellular domain contains a 266–amino acid stretch of homology to CAs. Although 11 of 19 active site residues are conserved, only 1 of 3 His residues that coordinate with zinc is conserved, making it unlikely that it has any CA activity. The murine homologue of this gene is expressed in developing brain, and both alleles are found defective in mouse L cells. Inactivating the CA-like domain, the putative receptor for some small molecule ligand, is thought to inactivate the tumor suppressor gene.

UNRELATED CARBONIC ANHYDRASES The periplasmic CA from green algae *Chlamydomonas reinhardtii* (141) is homologous to the animal CAs, with conservation of all of the active site residues thought to be critical for catalysis including 92, 94, 96, 106, 107, 117, 119, and 199 (see Figure 3). Thus, it appears to be related to the animal CAs and to be derived from the same common ancient ancestral gene.

For this reason, it came as a surprise when the recently determined sequences deduced from the cDNAs from spinach (142, 143) and from pea chloroplasts (144) were completely unrelated to CAs in the animal kingdom. This was even more surprising since the spinach enzyme has been characterized as a zinc-containing, high-activity enzyme with nearly 50% the activity of CA II and as having some affinity for sulfonamide inhibitors. It would appear that the plant kingdom achieved a solution to the need for CA activity by a completely different evolutionary strategy than that used by Chlamydomonas and all the members of the animal kingdom so far studied.

GENE ORGANIZATION AND CHROMOSOME LOCALIZATION

The structures of human genes for the CA I, II, III, IV, and VII (Figure 4) are similar in possessing six introns that separate exons 1–7. However, the human CA I gene has two additional noncoding exons called exon 1a and 1b, at the 5′ end of the gene (145), with 36 kb between exon 1a and the first coding exon, 1c.

The human CA IV gene also has an additional exon (115) (exon 1a) that encodes the signal sequence. Exons 1b through 7 encode the remaining coding sequences and the 3′ end untranslated region. The positions of introns 3, 4, 5, and 6 are identical with the corresponding positions of introns in the genes for the soluble CA isozymes (CA I, II, III, and VII). However, the positions of introns 1b and 2 in CA IV differ from the positions of the corresponding introns in genes for the soluble isozymes.

The genes for human CAs I-VII have been assigned to chromosomes 1, 8, 16, and 17. The CA I, II, and III genes (CA1, CA2, CA3) are clustered in a stretch of about 180 kb on chromosome 8q22 in the order of CA1, CA3, CA2. The CA II and CA III genes are transcribed in the same direction and opposite to that of CA I (146). CA IV was assigned to 17q23 (115), and CA VI was assigned to chromosome 1p36.22–33 (147). CA V was recently assigned to chromosome 16 (43), and CA VII was previously mapped on 16q22 (48).

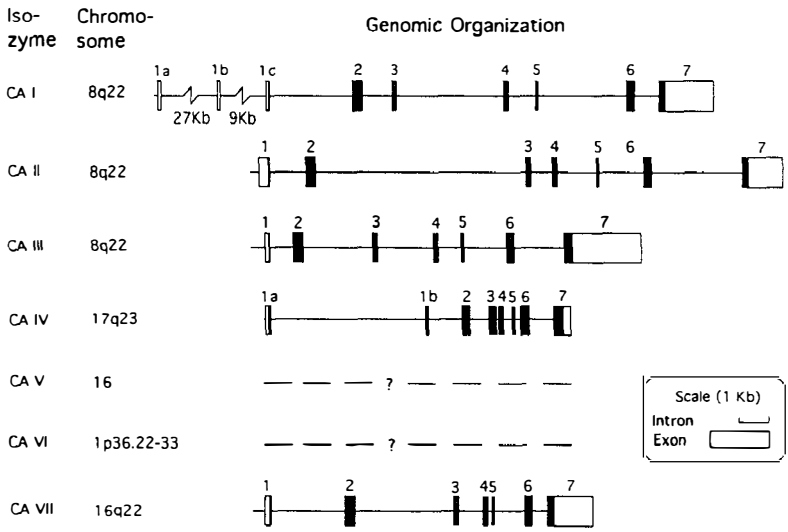

Figure 4 Structures and chromosomal localizations of human carbonic anhydrase isozyme genes. Numbered exons are indicated by boxes in which the coding regions are filled in black. The scale for exons is double that for introns. Sources: Human CA I (145); CA II (155); CA III (112); CA IV (115); CA V (88); CA VI (89); CA VII (48).

CARBONIC ANHYDRASE DEFICIENCY DISEASES

CA II Deficiency Syndrome

DISCOVERY OF CA II DEFICIENCY AND ITS IMPACT To date, the only known disease attributable to a deficiency of CA is the CA II deficiency syndrome (11), which is associated with osteopetrosis with renal tubular acidosis and cerebral calcification (McKusick catalog #259730) (148–151). In 1983, Sly et al (152) reported that the three affected members of the original American family with this disorder (150) lacked CA II in their erythrocytes, and that their normal-appearing parents and many of their first degree relatives had half-normal levels of CA II. CA I, the other CA isozyme normally present in erythrocytes, was present in normal amounts. These observations led them to propose that CA II deficiency was the primary defect in this newly recognized metabolic disorder. Since then, more than 50 patients with this syndrome have been reported, all of whom were shown to have CA II deficiency. Detailed clinical manifestations and pathogenesis are reviewed in 11, 12, 153, and 154.

This discovery attracted great interest for several reasons. First, it provided a biochemical explanation for one of several inherited forms of osteopetrosis, a method to distinguish patients with this form of osteopetrosis from those with other forms, and to screen for heterozygous carriers of this recessive form of osteopetrosis. Second, at a more fundamental level, it provided clear genetic evidence for a role of CA in bone resorption, and specifically implicated isozyme II in the process. Third, it allowed the distinct individual roles of CA II and CA IV in HCO_3^- reabsorption and acidification by the proximal renal tubules in the kidney to be delineated. Fourth, it clearly demonstrated an essential role for CA II in supporting H^+ secretion in the distal parts of the nephron that mediate distal urinary acidification. Fifth, the mental retardation and brain calcification seen in over 90% of affected patients demonstrated the importance of CA II (already known to be expressed in, and limited to, oligodendrocytes in brain) in normal brain development. Finally, the initial report appeared in April, 1983 (152), five months before the symposium (1) celebrating the 50th anniversary of the discovery of CAs, and the five decades of research that followed from that discovery. Naturally, the report that CA II deficiency is the basis for a newly recognized metabolic disorder affecting bone, brain, and kidney added considerable excitement to the anniversary celebration (1).

MOLECULAR GENETICS OF CA II DEFICIENCY Seven different mutations in the CA II structural gene have been identified by PCR amplification of genomic DNA from patients with this disorder (Figure 5). The first mutation was identified in a Belgian patient homozygous for a C-to-T transition in exon 3,

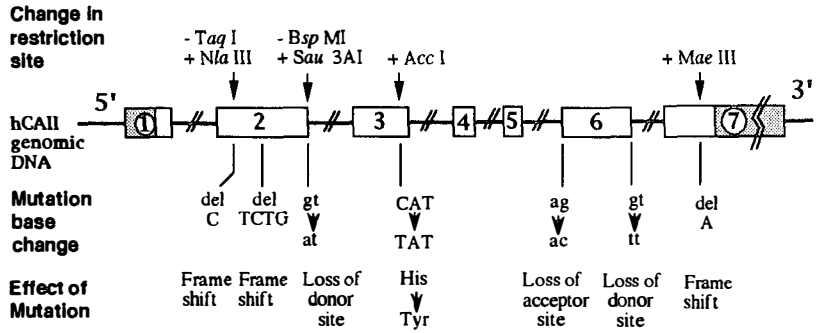

Figure 5 Seven structural mutations found to date in the human CA II gene. The + or − signs indicate the restriction sites introduced (+) or removed (−) by the mutation. Capital letters and the lower cases represent base pairs in exons and introns respectively. A gt→at change in the 5′ end of intron 2 destroyed a splice junction donor site. This is the commonest mutation in Arabic patients (159). A C→G transition in exon 3 results in a replacement of the conserved histidine at position 107 with tyrosine (His107Tyr). This mutation was identified in a homozygous Belgian patient (155), a homozygous Italian patient (PY Hu, P Strisciuglio, J Ciccolella, and WS Sly, unpublished data), and also as one of two mutations in the three American sisters who were compound heterozygotes. Their second mutation is an A→C transversion at the 3′ end of intron 5, which destroys a splice junction acceptor site (156). A single-base deletion in the coding region of exon 7 results in a frameshift at codon 227, which changes the next 12 amino acids and introduces a UGA stop codon 22 amino acids earlier than in the normal enzyme. This mutation was found to be common in Hispanic patients from the Caribbean islands (160, 161). Two different deletions in exon 2 and a gt→tt change in the 5′ end of intron 6 were discovered recently in two Italian families and one American family (PY Hu, P Strisciuglio, J Ciccolella, and WS Sly, unpublished data).

which results in replacement of the conserved histidine at position 107 with tyrosine (His107Tyr) (155). The three affected sisters in the American family in which CA II deficiency was first reported were also found to have this mutation. However, they were compound heterozygotes, having inherited the His107Tyr mutation from their mother and a splice acceptor mutation in the 3′ end of intron 5 from their father (156). Neither the Belgian patient nor the American patients were mentally retarded, as were most subsequently reported patients. Frequent skeletal fractures were the most disabling manifestation of this disease (157). When the CA II cDNA containing the His107Tyr mutation was expressed in *E. coli,* some CA activity was detected. These experiments led to the suggestion that a small amount of residual CA II activity in patients with the His107Tyr mutation may allow them to escape mental retardation (156). Detailed kinetic analysis of the mutant His107Tyr CA II isolated from overexpressing bacteria was reported by Tu et al (158).

The third structural gene mutation identified is a splice junction mutation at the 5′ end of intron 2, which was found in patients from Kuwait, Saudi Arabia, Algeria, and Tunisia (159), and all of the patients of Arabic descent

so far studied. More than 75% of the CA II-deficient patients so far recognized have been Arabic (157) and have been severely affected. In these patients with the "Arabic mutation," mental retardation and metabolic acidosis were prominent, while bone fractures were less frequent (157, 159).

A frameshift mutation resulting from a single-base deletion in the coding region of exon 7 was found in a mildly affected Hispanic girl, who is the only patient reported so far without renal tubular acidosis (160, 161). This single-base deletion results in a frameshift at codon 227 that changes the next 12 amino acids and introduces a UGA stop codon at codon 239. The truncated enzyme resulting from the mutation is 22 amino acids shorter than the 260 amino acids in normal CA II and is inactive. However, when expressed in bacteria, the mutant allele produced 0.05% of the activity expressed by the normal allele. This mutant enzyme activity resides in a small fraction of near-normal size enzyme (29 kDa), which had about 10% of normal specific activity. Protein sequencing showed that the first 11 amino acids following the frameshift mutation were abnormal in the 29-kDa mutant protein, as predicted, after which the reading frame was restored to normal. The last 23 amino acids of the 29-kDa mutant protein were the same as in normal CA II. These results can be explained by a ribosomal −1 translational frameshift that restores the reading frame 11 codons after the original mutation and allows completion of full-length CA II. Subsequently referred patients from seven independent Hispanic families, all derived from Caribbean ancestors, were found by sequencing or restriction site analysis to be homozygous for the same mutation. However, some of these Hispanic patients had severe clinical manifestations, including severe renal tubular acidosis, anemia, and hepatosplenomegaly. The basis for the wide clinical variability in these patients is not clear. However, these findings raise the interesting possibility that individual variation in efficiency of frameshift suppression could contribute to clinical heterogeneity among patients with identical frameshift mutations.

Recently, three new mutations were discovered in the CA II structural gene of CA II–deficient patients (PY Hu, P Strisciuglio, J Ciccolella, and WS Sly, unpublished data). Two deletion mutations were found in exon 2. A four base pair deletion was identified in an American family, and a single base deletion in an Italian family. Two other Italian families were studied recently, in one of which the CA II–deficient patient was homozygous for the already described His107Tyr mutation. In the other family, the CA II–deficient patient was homozyous for a splice junction mutation at the 5′ end of intron 6 (Figure 5).

CA I Deficiency Causes No Disease

The first report of human CA deficiency was a deficiency of CA I in a family originating from the Greek island of Icaria (163). However, except for the reduction in red cell CA I to trace levels, the individuals homozygous for the

deficiency gene in this family had no hematological or other abnormalities. Recently, it was reported that CA I–deficient members of this family had a missense mutation (Arg246His) in exon 7 of their CA I gene (164). This mutation is the probable cause of the CA I deficiency. Arg246 is not only conserved among all seven human CA isozymes (Figure 3) but is also present in all animal CAs and even in the CA-related proteins (13). This conservation led to the suggestion that Arg246 plays a critical role in the intramolecular structures of all CA molecules, and the substitution of His at this position results in an unstable molecule (164).

Even though CA I normally contributes to CA activity in vivo in humans, unless some low level activity of the Arg246His CA I is present in nonerythroid tissues, other CA isozymes or alternative processes can apparently substitute for the function of CA I when it is absent. The failure to find any clinical abnormalities in CA I deficiency led to the conclusion that the earlier report of a partial deficiency of CA I in a patient with distal renal tubular acidosis and deafness was merely a coincidental association (152).

A survey of red cell CA I concentrations in 3376 individuals in a Japanese population revealed that 25 had about half the normal level of CA I (165).

Other CA Genes in Search of a Disease

Will other CA deficiencies be found, or will the loss of other CA genes be harmless, as is the case for CA I? We hypothesize that loss of certain other CA genes would be quite deleterious. For example, CA IV is responsible for most of the bicarbonate reclamation by kidney. Thus, the phenotype of CA IV deficiency should include proximal renal tubular acidosis, at the minimum. How serious the loss would be of CA IV from brain, lung, eye, heart, and skeletal muscle would determine whether CA IV deficiency is compatible with life, and if so, what other handicaps such patients might have.

CA V deficiency may also be deleterious. Loss of CA V would probably lead to impairment of gluconeogenesis and ureagenesis. The defect in gluconeogenesis might produce fasting hypoglycemia, and the defect in ureagenesis, hyperammonemia. Many patients with unexplained fasting hypoglycemia or hyperammonemia or both are considered potential candidates for CA V deficiency. The availability of the cDNA and genomic sequences for both CA IV and CA V make it possible to examine DNA for mutations in patients suspected of having these deficiencies.

It seems less likely that loss of CA VI would produce a debilitating disease than loss of CA IV or CA V. However, if it does indeed have an important role in pH regulation in the upper alimentary tract as suggested (123), its loss could be significant.

Another difficult phenotype to predict is that of CA III deficiency. Despite the abundance of CA III in red skeletal muscle, its precise role in, and import-

ance to, muscle is still unclear. Is it in muscle for CO_2 hydration—or does it have another more important role? Also intriguing is the role of CA III in adipose tissue, and its apparent decline with the onset of obesity (108). It is difficult to anticipate what might happen in adipose tissue with loss of CA III. It seems likely that targeted mutagenesis in embryonic stem cells will provide murine models for individual deficiencies of each of the CAs in the next few years. Although these models will be important, the murine model does not always have the same features as the human disease. This is obvious from the CA II–deficient mouse (discussed below) that lacks the bone and brain findings of human CA II deficiency.

ANIMAL MODELS OF CA DEFICIENCY

CA I Deficiency in the Pigtail Macaque

The pigtail macaque has a common mutation that produces CA I deficiency in homozygous animals (166, 167). The mutation (–85C→G) creates an AUG codon 6 bp 5′ of the end of exon 1a and creates an upstream open reading frame (ORF) terminating 5 bp from the normal AUG (13). This mutation is of unusual interest as an example of an upstream ORF inhibiting translation from a downstream AUG, as an example of a nondeleterious mutation that occurred in a founder population prior to the Pleistocene glaciation, and because it has a peculiar *cis* negative regulatory effect on CA II expression, even though the CA II promoter is 100 kb away from the CA I mutation in exon 1a of the CA I gene. However, this is not a disease model, as CA I deficiency produces no disease in the pigtail macaque, as is also true in humans.

Mouse CA II Deficiency

A CA II–deficiency allele was produced in the mouse by chemical mutagenesis using ethylnitrosourea (169). Surprisingly, the homozygous CA II–deficient mice showed neither osteopetrosis nor cerebral calcification. These mice did exhibit growth impairment and a defect in renal acidification, and they were later found to develop calcifications in blood vessels with advanced age (170). The mutation was identified as a C-to-T substitution introducing a stop codon in place of Gln155 (Gln155X) (JS Platero, WS Sly, unpublished observations).

It is unclear why the CA II–deficient mouse lacks some important features of the human disease. Nonetheless, the absence of CA II made possible precise histochemical localization studies that clarify the distribution of membrane-associated CA IV in the murine kidney (171) and the eye (172). Brechue et al (173) used cell fractionation techniques to define the subcellular distribution of CA IV in the CA II–deficient mouse and also characterized its renal acidification abnormalities. Others (174) have used the CA II–deficient mouse in

seizure studies and found that CA II deficiency confers increased resistance to seizure-inducing stimuli. Biesecker et al (175) studied the effects of transplantation of CA II–deficient mice with normal bone marrow, and of normal mice with marrow from CA II–deficient animals. The latter suggested that the renal acidification defect, and the markedly increased sensitivity of these animals to acid loading, result from both the erythrocyte and the renal CA II deficiency.

Two groups have reached different conclusions regarding the oligodendrocyte findings in brain and the neuropathology. Ghandour et al (176) made detailed morphological and histochemical analyses of oligodendrocytes and astrocytes and concluded that there were no abnormalities in one-year-old CA II–deficient mice. By contrast, Cammer et al (177) found evidence of shrunken oligodendrocytes in white matter and gray matter, and increased abundance of astrocytes in the white matter. Possibly these differences are due to differences in age or diet. Another possibility is uncontrolled genetic differences between CA II–deficient mice in different laboratories. In many cases, the CA II mutation has not been removed by outbreeding from the heavily mutagenized strains in which it was induced. Genetic differences between strains developed from the original CA II–deficient mice by different laboratories could be very significant.

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