1 Carbonic Anhydrases: Catalytic and Inhibition Mechanisms, Distribution and Physiological Roles

Claudiu T. Supuran

CONTENTS

At least 14 different α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms have been isolated in higher vertebrates, wherein these zinc enzymes play crucial physiological roles. Some of these isozymes are cytosolic (CA I, CA II, CA III and CA VII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), one is mitochondrial (CA V) and one is secreted in the saliva (CA VI). Three acatalytic forms are also known, designated CA-related proteins (CARPs): CARP VIII, CARP X and CARP XI. Representatives of the β- and γ-CA family are highly abundant in plants, bacteria and archaea. These enzymes are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate, and at least α-CAs possess a high versatility, being able to catalyze other different hydrolytic processes, such as the hydration of cyanate to carbamic acid or of cyanamide to urea; aldehyde hydration to *gem*-diols; hydrolysis of carboxylic or sulfonic acids esters; as well as other less investigated hydrolytic processes, such as hydrolysis of halogeno derivatives and

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arylsulfonyl halides. It is not known whether the reactions catalyzed by CAs other than the hydration of CO_2/dehydration of HCO_3^- have physiological relevance in systems in which these enzymes are present. The catalytic mechanism of α-CAs is understood in great detail. The active site consists of a Zn(II) ion coordinated by three histidine residues and a water molecule/hydroxide ion. The latter is the active species, acting as a potent nucleophile. For β- and γ-CAs, the zinc hydroxide mechanism is valid too, although at least some β-class enzymes do not have water directly coordinated to the metal ion. CAs are inhibited primarily by two main classes of inhibitors: the metal-complexing inorganic anions (such as cyanide, cyanate, thiocyanate, azide and hydrogensulfide) and the unsubstituted sulfonamides possessing the general formula RSO_2NH_2 (R = aryl, hetaryl, perhaloalkyl). Several important physiological and physiopathological functions are played by the CA isozymes, which are present in organisms at all levels of the phylogenetic tree. Among these functions are respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs, biosynthetic reactions, such as the gluconeogenesis and urea synthesis (in animals) and CO_2 fixation (in plants and algae). The presence of these ubiquitous enzymes in so many tissues and in so many different isoforms makes them useful to design inhibitors or activators that have biomedical applications.

1.1 INTRODUCTION

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes and encoded by three distinct evolutionarily unrelated gene families: (1) α-CAs (in vertebrates, bacteria, algae and cytoplasm of green plants), (2) β-CAs (predominantly in bacteria, algae and chloroplasts of both mono- and dicotyledons) and (3) γ-CAs (mainly in archaea and some bacteria) (Hewett-Emmett 2000; Krungkrai et al. 2000; Chirica et al. 1997; Smith and Ferry 2000; Supuran and Scozzafava 2000, 2002; Supuran et al. 2003). In higher vertebrates, including humans, 14 α-CA isozymes or CA-related proteins (CARPs) have been described (Table 1.1), with very different subcellular localizations and tissue distributions (Hewett-Emmett 2000; Supuran and Scozzafava 2000, 2002; Supuran et al. 2003). There are several cytosolic forms (CAs I–III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) and a secreted CA isozyme (CA VI) (Supuran and Scozzafava 2000, 2002; Supuran et al. 2003). These enzymes catalyze a very simple physiological reaction, the interconversion of the carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues and organs, biosynthetic reactions (such as gluconeogenesis and lipoid and urea synthesis), bone resorption, calcification, tumorigenicity and many other physiological or pathological processes (Hewett-Emmett 2000; Supuran and Scozzafava 2000, 2002; Supuran et al. 2003). Many of these isozymes are important targets for the design of inhibitors with clinical applications.

In addition to the physiological reaction — the reversible hydration of CO_2 to bicarbonate (Equation 1.1, Figure 1.1) — α-CAs catalyze a variety of other reactions,

TABLE 1.1
Higher-Vertebrate α-CA Isozymes, Their Relative CO_2 Hydrase Activity, Affinity for Sulfonamide Inhibitors and Subcellular Localization

Isozyme	Catalytic Activity (CO_2 Hydration)	Affinity for Sulfonamides	Subcellular Localization
CAI	Low (10% of that of CA II)	Medium	Cytosol
CA II	High	Very high	Cytosol
CA III	Very low (0.3% of that of CA II)	Very low	Cytosol
CA IV	High	High	Membrane bound
CA V	Moderate-high[a]	High	Mitochondria
CA VI	Moderate	Medium-low	Secreted into saliva
CA VII	High	Very high	Cytosol
CARP VIII	Acatalytic	[b]	Cytosol
CA IX	High	High	Transmembrane
CARP X	Acatalytic	[b]	Cytosol
CARP XI	Acatalytic	[b]	Cytosol
CA XII	Low	High	Transmembrane
CA XIII	Moderate	High	Cytosol
CA XIV	High	High	Transmembrane

[a] Moderate at pH 7.4; high at pH 8.2 or higher pH.
[b] The native CARP isozymes do not contain Zn(II), so that their affinity for the sulfonamide inhibitors has not been measured. By site-directed mutagenesis it is possible to modify these proteins and transform them into enzymes with CA-like activity, which probably are inhibited by sulfonamides, but no detailed studies on this subject are available at present (see Chapter 2 for details on CARPs).

such as hydration of cyanate to carbamic acid or of cyanamide to urea (Equation 1.2 and Equation 1.3, Figure 1.1); aldehyde hydration to *gem*-diols (Equation 1.4, Figure 1.1); hydrolysis of carboxylic or sulfonic acid esters (Equation 1.5 and Equation 1.6, Figure 1.1); as well as other less-investigated hydrolytic processes, such as those described by Equation 1.7 to Equation 1.9 in Figure 1.1 (Briganti et al. 1999; Guerri et al. 2000; Supuran et al. 1997, 2003; Supuran and Scozzafava 2000, 2002). The previously reported phosphatase activity of CA III was recently proved to be an artefact (Kim et al. 2000). It is unclear whether α-CA catalyzed reactions other than CO_2 hydration have physiological significance. To date, x-ray crystal structures have been determined for six α-CAs (isozymes CA I to CA V and CA XII; Stams and Christianson 2000) as well as for representatives of the β- (Mitsuhashi et al. 2000) and γ-CA families (Kisker et al. 1996; Smith and Ferry 2000; Iverson et al. 2000).

1.2 CATALYTIC AND INHIBITION MECHANISMS OF CAs

1.2.1 α-CAs

The Zn(II) ion of CAs is essential for catalysis (Lindskog and Silverman 2000; Christianson and Fierke 1996; Bertini et al. 1982; Supuran et al. 2003). X-ray

$$O{=}C{=}O + H_2O \Leftrightarrow HCO_3^- + H^+ \quad (1.1)$$

$$O{=}C{=}NH + H_2O \Leftrightarrow H_2NCOOH \quad (1.2)$$

$$HN{=}C{=}NH + H_2O \Leftrightarrow H_2NCONH_2 \quad (1.3)$$

$$RCHO + H_2O \Leftrightarrow RCH(OH)_2 \quad (1.4)$$

$$RCOOAr + H_2O \Leftrightarrow RCOOH + ArOH \quad (1.5)$$

$$RSO_3Ar + H_2O \Leftrightarrow RSO_3H + ArOH \quad (1.6)$$

$$ArF + H_2O \Leftrightarrow HF + ArOH \quad (1.7)$$

(Ar = 2,4-dinitrophenyl)

$$PhCH_2OCOCl + H_2O \Leftrightarrow PhCH_2OH + CO_2 + HCl \quad (1.8)$$

$$RSO_2Cl + H_2O \Leftrightarrow RSO_3H + HCl \quad (1.9)$$

(R = Me; Ph)

FIGURE 1.1 Reactions catalyzed by α-CAs. (Reproduced from Supuran, C.T. et al. (2003) *Medicinal Research Reviews* **23**, 146–189, John Wiley & Sons. With permission.)

crystallographic data show that the metal ion is situated at the bottom of a 15-Å-deep active-site cleft (Figure 1.2), coordinated by three histidine residues (His 94, His 96 and His 119) and a water molecule/hydroxide ion (Christianson and Fierke 1996; Stams and Christianson 2000). The zinc-bound water is also engaged in hydrogen bond interactions with the hydroxyl moiety of Thr 199, which in turn is bridged to the carboxylate moiety of Glu 106. These interactions enhance the nucleophilicity of the zinc-bound water molecule and orient the substrate (CO_2) in a location favorable for nucleophilic attack (Figure 1.3; Lindskog and Silverman 2000; Stams and Christianson 2000; Supuran et al. 2003). The active form of the enzyme is the basic one, with hydroxide bound to Zn(II) (Figure 1.3A; Lindskog and Silverman 2000). This strong nucleophile attacks the CO_2 molecule bound in a hydrophobic pocket in its neighborhood (the substrate-binding site comprises residues Val 121, Val 143 and Leu 198 in human isozyme CA II — Christianson and Fierke 1996; Figure 1.3B), leading to the formation of bicarbonate coordinated to Zn(II) (Figure 1.3C). The bicarbonate ion is then displaced by a water molecule and liberated into solution, forming the acid form of the enzyme, with water coordinated to Zn(II) (Figure 1.3D), which is catalytically inactive (Lindskog and Silverman 2000; Christianson and Fierke 1996; Bertini et al. 1982; Supuran et al. 2003). To regenerate the basic form A, a proton transfer reaction from the active site to the environment occurs, which might be assisted either by active-site residues (such as His 64 — the proton shuttle in isozymes I, II, IV, VII and IX, among others; see Figure 1.2 for isozyme II) or by buffers present in the medium. The process is schematically represented by Equation 1.10 and Equation 1.11:

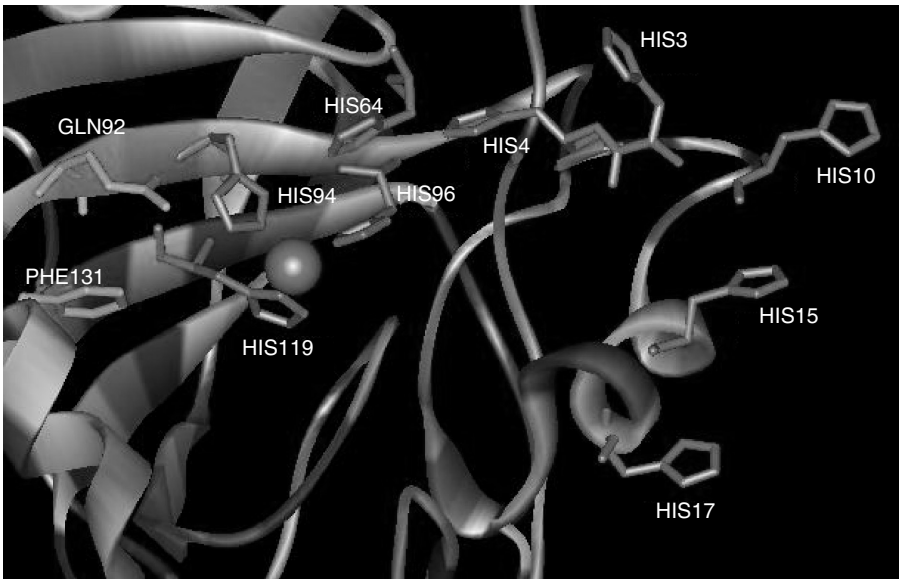


FIGURE 1.2 (See color insert following page 148.) hCA II active site. The Zn(II) ion (central pink sphere) and its three histidine ligands (in green, His 94, His 96, His 119) are shown. The histidine cluster, comprising residues His 64, His 4, His 3, His 17, His 15 and His 10, is also shown, as this is considered to play a critical role in binding activators of the types **6** to **14** reported in the chapter as well as the carboxyterminal part of the anion exchanger AE1. The figure was generated from the x-ray coordinates reported by Briganti et al. (1997) (PDB entry 4TST). (Reproduced from Scozzafava, A. and Supuran, C.T. (2002) *Biorganic Medicinal Chemistry Letters* **12**, 1177–1180. With permission from Elsevier.)

$$EZn^{2+} - OH^- + CO_2 \Leftrightarrow EZn^{2+} - HCO_3^- \overset{H_2O}{\Leftrightarrow} EZn^{2+} - OH_2 + HCO_3^- \quad (1.10)$$

$$EZn^{2+} - OH_2 \Leftrightarrow EZn^{2+} - HO^- + H^+ \quad (1.11)$$

The rate-limiting step in catalysis is the second reaction, i.e., the proton transfer that regenerates the zinc hydroxide species of the enzyme (Lindskog and Silverman 2000; Christianson and Fierke 1996; Bertini et al. 1982; Supuran et al. 2003). In the catalytically very active isozymes, such as CA II, CA IV, CA V, CA VII and CA IX, the process is assisted by a histidine residue (His 64) at the entrance of the active site, as well as by a cluster of histidines (Figure 1.2) that protrudes from the rim of the active site to the surface of the enzyme, assuring a very efficient proton transfer process for CA II, the most efficient CA isozyme (Briganti et al. 1997). This also explains why CA II is one of the most active enzymes known (with a $k_{cat}/K_m = 1.5 \times 10^8$ $M^{-1}s^{-1}$), approaching the limit of diffusion control (Lindskog and Silverman 2000; Christianson and Fierke 1996; Supuran et al. 2003), and also has important consequences for designing inhibitors that have clinical applications.

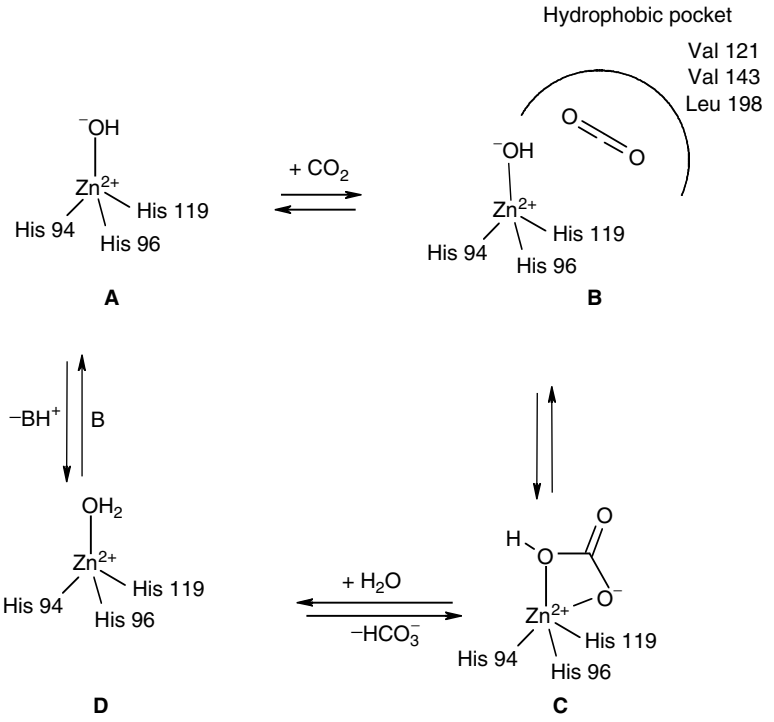


FIGURE 1.3 Schematic representation of the catalytic mechanism for the CA-catalyzed CO_2 hydration. The hypothesized hydrophobic pocket for the binding of substrates is shown schematically at Step B.

Two main classes of carbonic anhydrase inhibitors (CAIs) are known: the metal-complexing anions and the unsubstituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substituting the nonprotein zinc ligand (Equation 1.12, Figure 1.4) or add to the metal coordination sphere (Equation 1.13, Figure 1.4), generating trigonal-bipyramidal species (Bertini et al. 1982; Lindskog and Silverman 2000; Supuran et al. 2003; Supuran and Scozzafava 2000, 2002). Sulfonamides, the most important CAIs, bind in a tetrahedral geometry of the Zn(II) ion (Figure 1.4), in a deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds involving the residues Thr 199 and Glu 106, also participating in anchoring the inhibitor molecule to the metal ion. The aromatic/heterocyclic part of the inhibitor (R) interacts with hydrophilic and hydrophobic residues of the cavity. Anions might bind either in tetrahedral geometry of the metal ion or as trigonal-bipyramidal adducts, such as the thiocyanate adduct shown in Figure 1.4B (see also Chapter 7 for a detailed description of CA inhibition by anions; Lindahl et al. 1991; Stams and Christianson 2000; Abbate et al. 2002; Supuran et al. 2003).

X-ray crystallographic structures are available for many adducts of sulfonamide inhibitors with isozymes CA I, CA II and CA IV (see also Chapter 3; Lindahl et al. 1991; Stams et al. 1996; Stams and Christianson 2000; Abbate et al. 2002, 2003;

$$E\text{-}Zn^{2+}\text{-}OH_2 + I \Leftrightarrow E\text{-}Zn^{2+}\text{-}I + H_2O \quad \textbf{(substitution)} \quad (1.12)$$

Tetrahedral adduct

$$E\text{-}Zn^{2+}\text{-}OH_2 + I \Leftrightarrow E\text{-}Zn^{2+}\text{-}OH_2(I) \quad \textbf{(addition)} \quad (1.13)$$

Trigonal-bipyramidal adduct

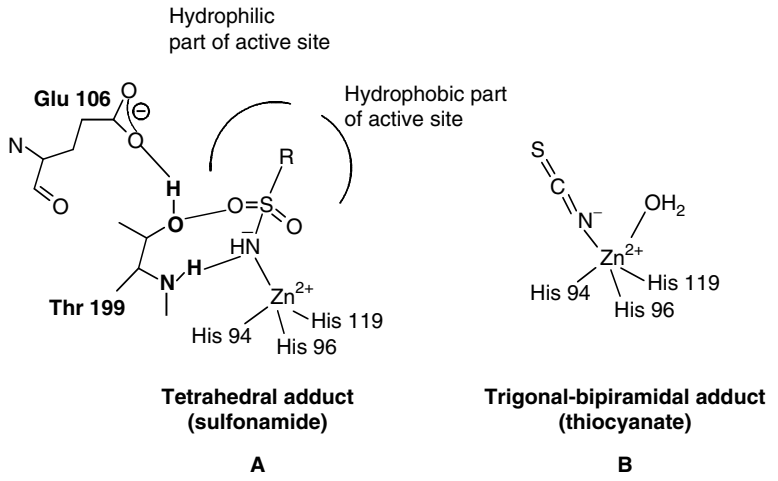


FIGURE 1.4 CA inhibition mechanism by sulfonamide (A) and anionic (B) inhibitors. In the case of sulfonamides, in addition to the Zn(II) coordination, an extended network of hydrogen bonds ensues, involving residues Thr 199 and Glu 106, whereas the organic part of the inhibitor (R) interacts with hydrophilic and hydrophobic residues of the cavity. For anionic inhibitors such as thiocyanate (B), the interactions between inhibitor and enzyme are much simpler.

Supuran et al. 2003). In all the adducts, the deprotonated sulfonamide is coordinated to the Zn(II) ion of the enzyme and its NH moiety participates in a hydrogen bond with the Oγ of Thr 199, which in turn is engaged in another hydrogen bond to the carboxylate group of Glu 106 (Lindahl et al. 1991; Stams et al. 1996; Stams and Christianson 2000; Abbate et al. 2002; Supuran et al. 2003). One of the oxygen atoms of the SO_2NH moiety also participates in a hydrogen bond with the backbone NH moiety of Thr 199. Figure 1.5 shows the crystal structures of the hCA II adducts with the simplest compounds incorporating a sulfamoyl moiety (sulfamide and sulfamic acid). The binegatively charged $(NH)SO_3^{2-}$ sulfamate ion and the mono-anion of sulfamide $NHSO_2NH_2^-$ bind to the Zn(II) ion within the enzyme active site (Abbate et al. 2002). These two structures provide some close insights into why this functional group (the sulfonamide group) appears to have unique properties for CA inhibition: (1) it exhibits a negatively charged, most likely monoprotonated nitrogen coordinated to the Zn(II) ion; (2) simultaneously, this group forms a hydrogen bond as donor to the oxygen Oγ of the adjacent Thr 199; and (3) a hydrogen bond is formed between one of the SO_2 oxygens and the backbone NH of Thr 199. The basic structural elements explaining the strong affinity of the sulfonamide moiety

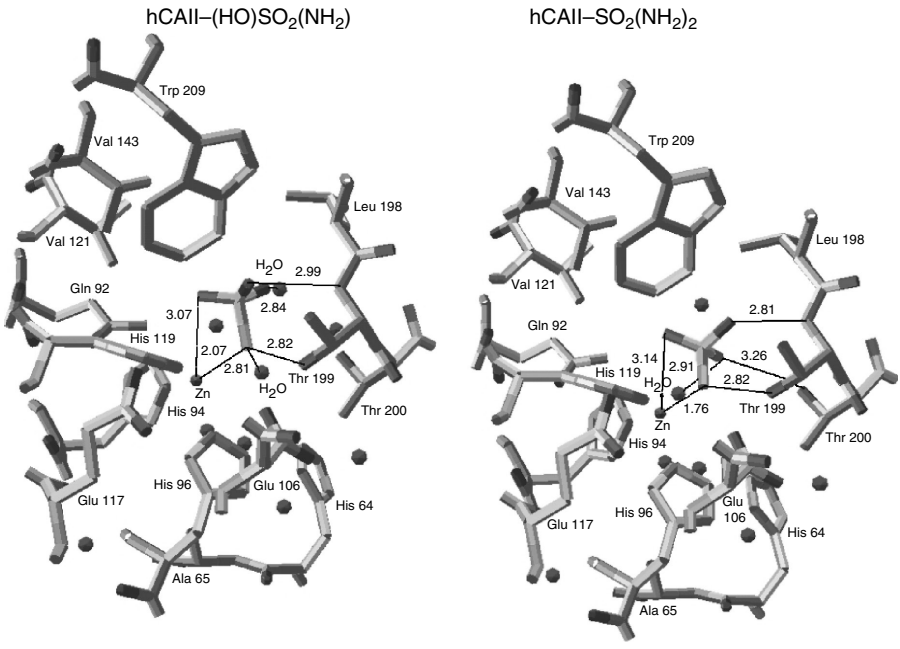


FIGURE 1.5 Adducts of hCA II with the simplest sulfonamides: sulfamic acid H_2NSO_3H, (left) and sulfamide $H_2NSO_2NH_2$ (right), determined by x-ray crystallography. (Reproduced from Supuran, C.T. et al. (2003) *Medicinal Research Reviews* **23**, 146–189, John Wiley & Sons. With permission.)

for the Zn(II) ion of CAs have been delineated in detail by using these simple compounds as prototypical CAIs (Briganti et al. 1996), without needing to analyze the interactions of the organic scaffold usually present in other inhibitors [generally belonging to the aromatic/heterocyclic sulfonamide class (Abbate et al. 2002)]. Despite important similarities in the binding of these two inhibitors to the enzyme to that of aromatic/heterocyclic sulfonamides of the type RSO_2NH_2 previously investigated, the absence of a C–SO_2NH_2 bond in sulfamide/sulfamic acid leads to a different hydrogen bond network in the neighborhood of the catalytical Zn(II) ion, which has been shown to be useful for the design of more potent CA inhibitors as drugs, possessing zinc-binding functions different from those of classical sulfonamides (Abbate et al. 2002).

The physiological function of the major red cell isozyme CA I (present in concentrations of up to 150 μM in the blood; Supuran et al. 2003) is unknown. Recently, the x-ray crystal structure of a natural mutant of CA I, i.e., CA I Michigan 1 (Figure 1.6), was reported. [The isozyme was isolated in three generations of a family of European Caucasians (Ferraroni et al. 2002a).] CA I Michigan 1 differs from wild-type CA I in a single amino acid residue present in the active-site cavity, i.e., Arg 67 instead of His 67 (Ferraroni et al. 2002a). This amino acid residue is located in an important region of the catalytic site, which is involved both in shuttling

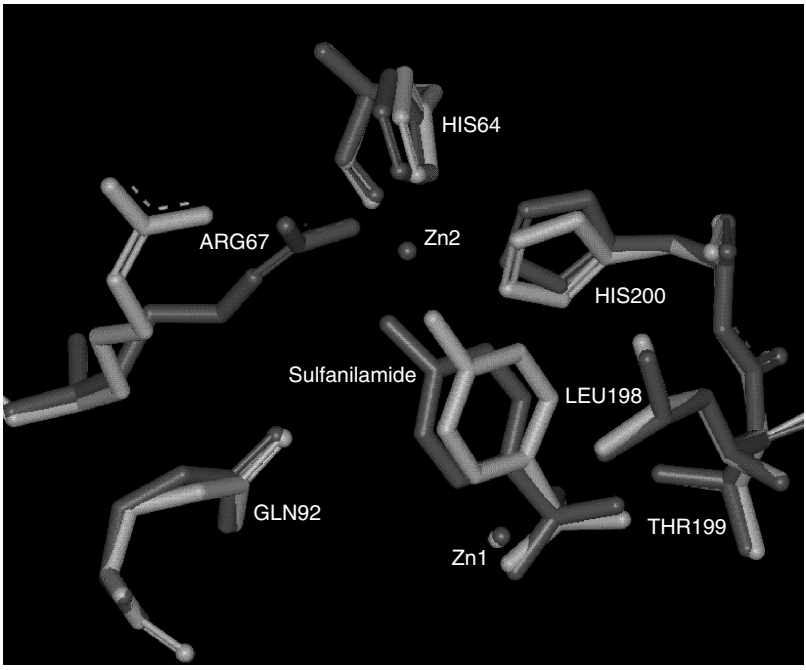


FIGURE 1.6 (See color insert.) Least-squares superimposition of the most relevant active site residues of the natural mutant CA I Michigan 1 (in yellow) and the CA I Michigan 1 $(Zn)_2$ adduct (in red) involved in sulfonamide inhibitor binding, with bound sulfanilamide, as determined by x-ray crystallography. The catalytic zinc ion is Zn1. (Reproduced from Supuran, C.T. et al. (2003) *Medicinal Research Reviews* **23**, 146–189, John Wiley & Sons. With permission.)

protons from the active cavity to the environment and in binding aromatic/heterocyclic sulfonamides, the classical, clinically important CAIs (Figure 1.6). The structure of the native mutant enzyme has been determined, as well as its adduct with a second zinc ion, which reveals the presence of a second metal-ion-binding site within the active cavity. Arg 67 appears to promote the binding of this second zinc ion to His 64, His 200 and itself (through one of the guanidino nitrogen atoms) (Ferraroni et al. 2002a). This second zinc ion bound to the active cavity is involved in the previously observed activation mechanism for substrate-specific α- and β-naphthyl acetate hydrolyses (Ferraroni et al. 2002a). Furthermore, this is the first example of a Zn(II) enzyme containing an arginine residue in the metal ion coordination sphere and the first CA isozyme that binds two metal ions within its active site (Ferraroni et al. 2002a). The crystal structures of sulfanilamide (4-aminobenzene sulfonamide) complexed to native hCA I Michigan 1 variant and to its $(Zn)_2$ adduct were also reported (Ferraroni et al. 2002b). Comparisons among these structures and the corresponding sulfonamide adduct of hCA I showed significant differences in the orientation of

the inhibitor molecule and in its interactions with active-site residues such as His 200, Thr 199, Leu 198, Gln 92 and Arg/His67, which are known to play important roles in substrate or inhibitor binding and recognition (Supuran et al. 2003; Ferraroni et al. 2002a). In CA I Michigan 1 a lengthening of the Zn–N1 sulfanilamide bond distance and a corresponding shortening of the distance between the sulfamido group and Thr 199 as compared with wild-type CA I were observed. When the second Zn(II) ion was present in the active site, the *p*-amino group and the aromatic ring of the inhibitor molecule appeared tilted toward Gln 92 and Arg 67, moving away from His 200 and Leu 198. The structural differences in inhibitor binding between the CA I isozyme and the CA I Michigan 1 variant showed that even a point mutation within the active site of a CA isozyme might have relevant consequences on the binding of inhibitors (Ferraroni et al. 2002a). This work opens a new direction for designing isozyme-specific CAIs.

The different types of interactions by which a sulfonamide CAI achieves very high affinity (in a low nanomolar range) for the CA active site, are illustrated in Figure 1.7 for a fluoro-containing inhibitor in early clinical development, PFMZ (4-methyl-5-perfluorophenylcarboximido-δ^2-1,3,4-thiadiazoline-2-sulfonamide; Abbate et al. 2003). The ionized sulfonamide moiety of PFMZ replaces the hydroxyl ion coordinated to Zn(II) in the native enzyme (Zn–N distance 1.95 Å), with the metal ion remaining in its stable tetrahedral geometry, being coordinated in addition to the sulfonamidate nitrogen by the imidazolic nitrogens of His 94, His 96 and His 119. The proton of the coordinated sulfonamidate nitrogen atom also makes a hydrogen bond with the hydroxyl group of Thr 199, which in turn accepts a hydrogen bond from the carboxylate of Glu 106. One of the oxygen atoms of the sulfonamide moiety makes a hydrogen bond with the backbone amide of Thr 199, whereas the other is semicoordinated to the catalytic Zn(II) ion (O–Zn distance 3.0 Å). The thiadiazoline ring of the inhibitor lies in the hydrophobic part of the active-site cleft, wherein its ring atoms make van der Waals interactions with the side chains of Leu 204, Pro 202, Leu 198 and Val 135 (Figure 1.7). The carbonyl oxygen of PFMZ makes a strong hydrogen bond with the backbone amide nitrogen of Gln 92 (of 2.9 Å), an interaction also evidenced for the acetazolamide–hCA II adduct. Besides Gln 92, two other residues, Glu 69 and Asn 67, situated in the hydrophilic half of the CA active site, make van der Waals contacts with the PFMZ molecule complexed to hCA II. But the most notable and unprecedented interactions seen in this complex are the hydrogen bonds network involving the exocyclic nitrogen atom of the inhibitor, two water molecules (Wat 1194 and Wat 1199) and a fluorine atom in the *meta* position belonging to the perfluorobenzoyl tail of PFMZ (Figure 1.7). Thus, a strong hydrogen bond (of 2.9 Å) is formed between the imino nitrogen of PFMZ and Wat 1194, which in turn makes a hydrogen bond with a second water molecule of the active site, Wat 1199 (distance 2.7 Å). The second hydrogen of Wat 1194 also participates in a weaker hydrogen bond (3.3 Å) with the carbonyl oxygen of PFMZ. The other hydrogen atom of Wat 1199 makes a weak hydrogen bond with the fluorine atom in Position 3 of the perfluorobenzoyl tail of PFMZ (Figure 1.7). Finally, a very interesting interaction is observed between the perfluorophenyl ring of PFMZ and the phenyl moiety of Phe 131, a residue critical for the binding of inhibitors with

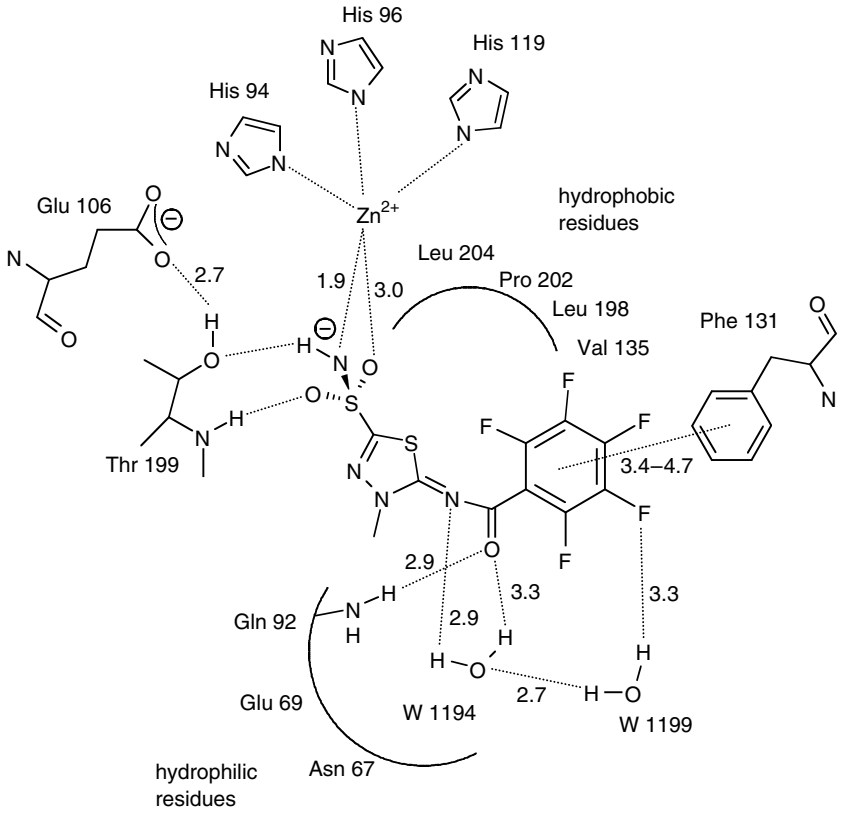
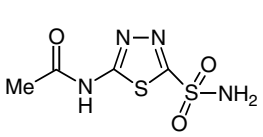


FIGURE 1.7 Schematic representation of the pentafluorobenzoyl analogue of methazolamide bound within the hCA II active site (figures represent distances in Å). (From Abbate, F. et al. (2003) *Journal of Enzyme Inhibition and Medicinal Chemistry*, **18**, 303–308. With permission.)

long tails to hCA II (Supuran et al. 2003). These two rings are almost perfectly parallel, situated at a distance of 3.4 to 4.7 Å. This type of stacking interactions has not been previously observed in a hCA II–sulfonamide adduct.

Since the report that sulfanilamide acts as a specific inhibitor of CA (Mann and Keilin 1940), four systemic sulfonamide CAIs have been developed and used clinically, mainly as antiglaucoma drugs, for some time: acetazolamide (**1.1**), methazolamide (**1.2**), ethoxzolamide (**1.3**) and dichlorophenamide (**1.4**) (Maren 1967; Supuran et al. 2003). As seen from the data in Table 1.2, Compound **1.1** to Compound **1.4** strongly inhibit several CA isozymes (such as CA I, CA II, CA IV, CA V, CA VII and CA IX), with affinities in the low nanomolar range for many of them. Recently, two new drugs have been introduced in clinical practice as topically acting sulfonamide CAIs — dorzolamide (**1.5**) and brinzolamide (**1.6**) — which also act as very potent inhibitors of most α-CA isozymes (Table 1.2). The design of sulfonamide CAIs as drugs is described in detail in Chapter 4.



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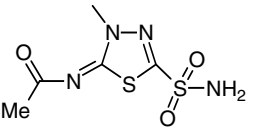

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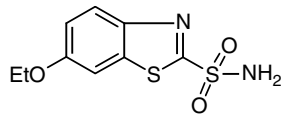

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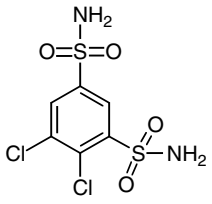

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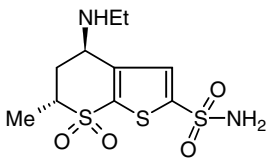

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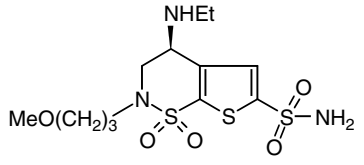

1.6

TABLE 1.2
Inhibition Data with Clinically Used Sulfonamides 1.1–1.6 against Several α-CA Isozymes of Human or Murine Origin

	K_I (nM)					
Isozyme	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**
hCA I	200	10	1	350	50,000	nt
hCA II	10	8	0.7	30	9	3
hCA III	3×10^5	1×10^5	5000	nt	8000	nt
hCA IV	66	56	13	120	45	45
mCA V	60	nt	5	nt	nt	nt
hCA VI	1100	560	nt	nt	nt	nt
mCA VII	16	nt	0.5	nt	nt	nt
hCA IX	25	27	34	50	52	37

Note: h = human; m: = murine isozyme; nt = not tested (no data available).

1.2.2 β-CAs

Many bacteria, some archaeas (such as *Methanobacterium thermoautotrophicum*), algae and chloroplasts of superior plants contain CAs belonging to the β-class (Smith and Ferry 1999, 2000; Kimber and Pai 2000; Mitsuhashi et al. 2000; Cronk et al. 2001). The principal difference between these enzymes and α-CAs is that β-CAs are usually oligomers, generally formed of two to six monomers of molecular weight 25 to 30 kDa each. The x-ray structures of four such β-CAs are available at the present time: (1) the enzyme isolated from the red alga *Porphyridium purpureum* (Mitsuhashi et al. 2000), (2) the enzyme from chloroplasts of *Pisum sativum* (Kimber

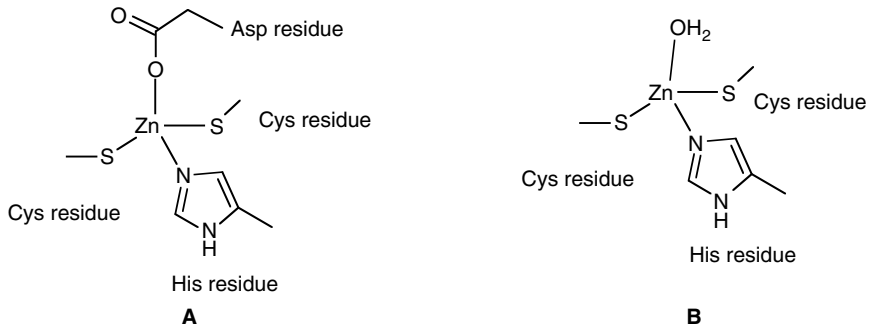

FIGURE 1.8 Schematic representation of the Zn(II) coordination sphere in β-CAs: **A:** *Porphyridium purpureum* [Mitsuhashi, S. et al. (2000) *Journal of Biological Chemistry* **275**, 5521–5526] and *Escherichia coli* [Cronk, J.D. et al. (2001) *Protein Science* **10**, 911–922] enzymes; **B:** *Pisum sativum* chloroplast and *Methanobacterium thermoautotrophicum* enzyme [Kimber, M.S., and Pai, E.F. (2000) *EMBO Journal* **15**, 2323–2330; Strop, P. et al. (2001) *Journal of Biological Chemistry* **276**, 10299–10305], as determined by x-ray crystallography.

and Pai 2000), (3) a prokaryotic enzyme isolated from *Escherichia coli* (Cronk et al. 2001) and (4) cab, an enzyme isolated from the archaeon *Methanobacterium thermoautotrophicum* (Strop et al. 2001).

The *Porphyridium purpureum* CA monomer is composed of two internally repeating structures folded as a pair of fundamentally equivalent motifs of an α/β domain and three projecting α-helices. The motif is very distinct from that of α- or γ-CAs. This homodimeric CA appeared like a tetramer with a pseudo 2-2-2 symmetry (Mitsuhashi et al. 2000). β-CAs are thus very different from the α-class enzymes. The Zn(II) ion is essential for catalysis in both families of enzymes, but its coordination is different and rather variable for β-CAs. Thus, in the prokaryotic β-CAs, the Zn(II) ion is coordinated by two cysteinate residues, an imidazole from a His residue and a carboxylate belonging to an Asp residue (Figure 1.8A), whereas the chloroplast enzyme has the Zn(II) ion coordinated by the two cysteinates, the imidazole belonging to a His residue and a water molecule (Figure 1.8B; Kimber and Pai 2000; Mitsuhashi et al. 2000; Cronk et al. 2001). The polypeptide chain folding and active-site architecture is clearly very different from those of α-CAs.

Because no water is directly coordinated to Zn(II) for some members of β-CAs (Figure 1.8A), the main question is whether the zinc hydroxide mechanism presented here for α-CAs is also valid for enzymes belonging to the β-family. A response to this question has been given by Mitsuhashi et al. (2000), who have proposed the catalytic mechanism shown in Figure 1.9.

As there are two symmetrical structural motifs in one monomer of the *Porphyridium purpureum* enzyme, resulting from two homologous repeats related to each other by a pseudo twofold axis, two Zn(II) ions are coordinated by the four amino acids mentioned. In this case, these pairs are Cys 149/Cys 403, His 205/His 459, Cys 208/Cys 462 and Asp 151/Asp 405 (Mitsuhashi et al. 2000). A water molecule is also present in the neighborhood of each metal ion, but it is not directly coordinated to it, forming a hydrogen bond with an oxygen belonging to the zinc ligand Asp

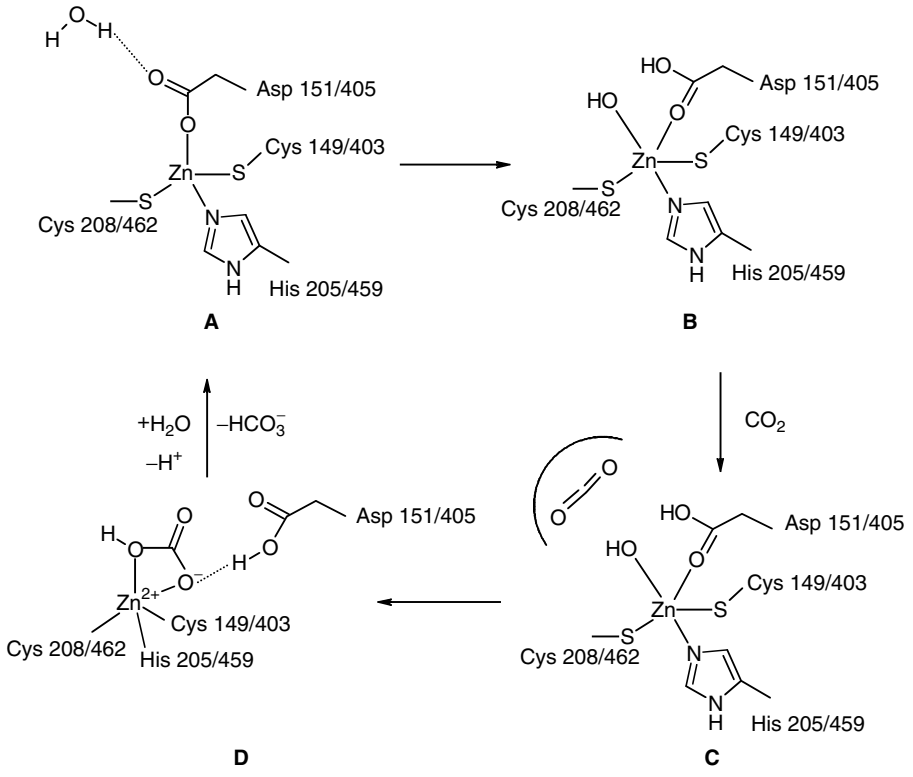

FIGURE 1.9 Proposed catalytic mechanism for prokaryotic β-CAs (*Porphyridium purpureum* enzyme numbering). (From Mitsuhashi, S. et al. (2000) *Journal of Biological Chemistry* **275**, 5521–5526.)

151/Asp 405 (Figure 1.9A). It is hypothesized that a proton transfer reaction might occur from this water molecule to the coordinated carboxylate moiety of the aspartate residue, generating a hydroxide ion, which might then be coordinated to Zn(II), which acquires a trigonal-bipyramidal geometry (Figure 1.9B). Thus, the strong nucleophile that might attack CO_2 bound within a hydrophobic pocket of the enzyme is formed (Figure 1.9C), with generation of bicarbonate bound to Zn(II) (Figure 1.9D). This intermediate is rather similar to the reaction intermediate proposed for the α-CA catalytic cycle (Figure 1.3C), except that for the β-class enzyme the aspartic acid residue originally coordinated to zinc is proposed to participate in a hydrogen bond with the coordinated bicarbonate (Figure 1.9D). In the last step, the coordinated bicarbonate is released into solution, together with a proton (no details available on this proton transfer process), the aspartate generated recoordinates the Zn(II) ion and the accompanying water molecule forms a hydrogen bond with it. The enzyme is thus ready for another cycle of catalysis.

The structure of the β-CA from the dicotyledonous plant *Pisum sativum* at a 1.93-Å resolution has also been reported (Kimber and Pai 2000). The molecule

assembles as an octamer with a novel dimer of dimers of dimers arrangement. The active site is located at the interface of two monomers, with Cys 160, His 220 and Cys 223 binding the catalytic zinc ion and Asp 162 (oriented by Arg 164), Gly 224, Gln 151, Val 184, Phe 179 and Tyr 205 interacting with acetic acid. The substrate-binding groups have a one-to-one correspondence with the functional groups in the α-CA active site, with the corresponding residues closely superimposable by a mirror plane. Therefore, despite differing folds, α- and β-CAs have converged on a very similar active-site design and are likely to share a common mechanism of action (Kimber and Pai 2000).

Cab exists as a dimer with a subunit fold similar to that observed in plant-type β-CAs. The active-site zinc ion was shown to be coordinated by the amino acid residues Cys 32, His 87 and Cys 90, with the tetrahedral coordination completed by a water molecule (Strop et al. 2001). The major difference between plant- and cab-type β-CAs is in the organization of the hydrophobic pocket (except for the zinc coordination). The structure also reveals a HEPES buffer molecule bound 8 Å away from the active-site zinc, which suggests a possible proton transfer pathway from the active site to the solvent (Strop et al. 2001). No structural data are currently available on the binding of inhibitors to this type of CAs, except that acetate coordinates to the Zn(II) ion of the *Pisum sativum* enzyme (Kimber and Pai 2000).

1.2.3 γ-CAs

Cam, the prototype of the γ-class CAs, has been isolated from the methanogenic archaeon *Methanosarcina thermophila* (Iverson et al. 2000). Crystal structures of zinc-containing and cobalt-substituted Cam have been reported in the unbound form and cocrystallized with sulfate or bicarbonate (Iverson et al. 2000).

Several features differentiate Cam from α- and β-CAs. The protein fold is composed of a left-handed β-helix motif interrupted by three protruding loops and followed by short and long α-helices. The Cam monomer self-associates as a homo-trimer with an approximate molecular weight of 70 kDa (Kisker et al. 1996; Iverson et al. 2000). The Zn(II) ion within the active site is coordinated by three histidine residues, as in α-CAs, but relative to the tetrahedral coordination geometry seen at the active site of α-CAs, the active site of this γ-CA contains additional metal-bound water ligands, so that the overall coordination geometry is trigonal-bipyramidal for the zinc-containing Cam and octahedral for the cobalt-substituted enzyme. Two of the His residues coordinating the metal ion belong to one monomer (Monomer A), whereas the third is from the adjacent monomer (Monomer B). Thus, the three active sites are located at the interface of pairs of monomers (Kisker et al. 1996; Iverson et al. 2000). The catalytic mechanism of γ-CAs is proposed to be similar to that of α-class enzymes (see Section 1.2.1; Kisker et al. 1996). Still, the finding that Zn(II) is not tetracoordinated as originally reported (Kisker et al. 1996) but pentacoordinated (Iverson et al. 2000), with two water molecules bound to the metal ion, demonstrates that much is still to be understood about these enzymes. At present, the zinc hydroxide mechanism is accepted as being valid for γ-CAs, as it is probable that an equilibrium exists between the trigonal-bipyramidal and the tetrahedral species of the metal ion in the active site of the enzyme.

Ligands bound to the active site have been shown to make contacts with the side chain of Glu 62 in a manner that suggests that this side chain to be probably protonated. In the uncomplexed zinc-containing Cam, the side chains of Glu 62 and Glu 84 appear to share a proton; additionally, Glu 84 exhibits multiple conformations. This suggests that Glu 84 might act as a proton shuttle, which is an important aspect of the reaction mechanism of α-CAs, for which a histidine active-site residue, usually His 64, generally plays this function (see Section 1.2.1). Anions such as bicarbonate or sulfate have been shown to bind to Cam (Iverson et al. 2000), but no information is available on its inhibition by sulfonamides.

1.2.4 Cadmium CA

X-ray absorption spectroscopy at the Zn K-edge indicates that the active site of the marine diatom *Thalassiosira weissflogii* CA (TWCA1) is strikingly similar to that of mammalian α-CAs. The zinc has three histidine ligands and a single water molecule, being quite different from the β-CAs of higher plants in which zinc is coordinated by two cysteine thiolates, one histidine and a water molecule (Cox et al. 2000). The diatom CA shows no significant sequence similarity with other CAs and probably represent an example of convergent evolution at the molecular level. In the same diatom, a rather perplexing discovery has been made — that of the first cadmium-containing enzyme, which is a CA-type protein (Lane and Morel 2000). Growth of the marine diatom *Thalassiosira weissflogii* under conditions of low zinc, typical of the marine environment, and in the presence of cadmium salts led to increased cellular CA activity, although the levels of TWCA1, the major intracellular Zn-requiring isoform of CA in *T. weissflogii*, remained low (Lane and Morel 2000). ^{109}Cd labeling comigrates with a protein band that shows this CA activity to be distinct from TWCA1 on native PAGE of the radiolabeled *T. weissflogii* cell lysates. The levels of the Cd protein were modulated by CO_2 in such a manner that they were consistent with the role of this enzyme in carbon acquisition. Purification of the CA-active fraction led to the isolation of a Cd-containing protein of 43 kDa, proving that *T. weissflogii* expresses a Cd-specific CA, which, particularly under conditions of Zn limitation can replace the Zn enzyme TWCA1 in its carbon-concentrating mechanism (Lane and Morel 2000).

1.3 DISTRIBUTION OF CAs

CAs were recently shown to be present in a multitude of prokaryotes, in which these enzymes play important functions, such as respiration, transport of carbon dioxide and photosynthesis (Smith and Ferry 2000). The possibility of developing CA-inhibitor-based antibiotics by inhibiting bacterial CAs present in pathogenic species raised much interest some years ago, with promising results in the use of ethoxzolamide for treating meningitis (Eickhoff and Nelson 1966; Nafi et al. 1990). This type of inhibition has also been exploited for developing selective culture media for other pathogenic bacteria, such as *Branhamella catarrhalis* (Nafi et al. 1990), in the presence of different *Neisseria* species. Some strains of *Pseudomonas*, *Staphylococcus*,

Streptococcus, *Serratia* and *Proteus* strongly express a gene product that was immunologically related to CA (Nafi et al. 1990). On the other hand, α-, β- and γ-CAs have been purified in many species of bacteria, such as *Neisseria* spp., *E. coli, Synechocystis* spp., *Acetobacterium woodi*, *Anabaena variabilis* and *Rhodospirillum rubrum*, but it is established that these enzymes are nearly ubiquitous in prokaryotes (Nafi et al. 1990, Chirica et al. 1997, 2001; Smith and Ferry 2000). Lindskog's group reported the isolation, purification and characterization of some α-CAs from pathogenic bacteria, such as *Helicobacter pylori* and *Neisseria gonorrhoeae* (Chirica et al. 1997, 2001; Elleby et al. 2001). Thus, the CA from *Helicobacter pylori* strain 26695 was toxic to *E. coli*, and therefore a modified form of the gene lacking a part that presumably encodes a cleavable signal peptide has been used for its expression. This truncated gene could be expressed in *E. coli*, yielding an active enzyme comprising 229 amino acid residues, with the amino acid sequence showing 36% identity with that of the enzyme from *N. gonorrhoeae* and 28% with that of hCA II (Elleby et al. 2001). The *H. pylori* CA was purified by sulfonamide affinity chromatography and its kinetic parameters for CO_2 hydration determined. Thiocyanate showed an uncompetitive inhibition pattern at pH 9, indicating that the maximal rate of CO_2 hydration is limited by proton transfer reactions between a zinc-bound water molecule and the reaction medium in analogy to higher-vertebrate α-CAs. The 4-nitrophenyl acetate hydrolase activity of the *H. pylori* enzyme was quite low, whereas the esterase activity against 2-nitrophenyl acetate as substrate was much better (Elleby et al. 2001). The kinetic properties of the CA isolated from *N. gonorrhoeae* (NGCA) as well as for some mutants of such enzymes have also been investigated by the same group (Chirica et al. 2001). Qualitatively, the enzyme shows the same kinetic behavior as that of the well-studied hCA II, suggesting a ping-pong mechanism with buffer as the second substrate. The ratio k_{cat}/K_m is dependent on two ionizations with pK_a values of 6.4 and 8.2, suggesting that His 66 in NGCA has the same function as a proton shuttle as does His 64 in hCA II. The kinetic defect in some NGCA mutants lacking His 66 can partially be overcome by some buffers, e.g., imidazole and 1,2-dimethylimidazole, which act as endogenous activators. The bacterial enzyme shows similar K_i values for the inhibitors cyanate, thiocyanate and azide as does hCA II, whereas cyanide and the sulfonamide ethoxzolamide are considerably weaker inhibitors of the bacterial enzyme than of hCA II (Chirica et al. 2001). Smith and Ferry (2000) recently published an excellent review on prokaryotic CAs.

The recent report on parasitic CAs by Krungkrai et al. (2000), who discovered the presence of at least two different α-CAs in *Plasmodium falciparum*, the malaria-provoking protozoan, opens new vistas to develop pharmacological agents based on CA inhibitors. Red cells infected by *Plasmodium falciparum* contain CA levels ca. twofold higher than those of normal red cells (Krungkrai et al. 2000). The three developmental forms of the asexual stages of the parasite (i.e., ring, trophozoite and schizont) were isolated from their host red cells and found to have stage-dependent CA activity. The enzyme was then purified to homogeneity and shown to have a M_r of 32 kDa, being active in monomeric form. (The human red cell enzyme was also purified for comparison with the parasite enzyme in this study; Krungkrai et al.

2000). The parasite enzyme activity was sensitive to well-known sulfonamide CAIs such as sulfanilamide and acetazolamide. The kinetic properties and the amino-terminal sequences of the purified enzymes from the parasite and host red cells were found to be different, indicating that the purified protein was a distinct enzyme, i.e., *P. falciparum* CA. In addition, the enzyme inhibitors showed antimalarial effect against *in vitro* growth of *P. falciparum*. This very important contribution shows that CAIs might be valuable drugs in the future to treat malaria.

In higher plants, algae and cyanobacteria, all members of the three CA families are present (Moroney et al. 2001). For example, analysis of the *Arabidopsis* database revealed that at least 14 different CAs are present in this plant, and six such enzymes are present in the unicellular green alga *Chlamydomonas reinhardtii* (Moroney et al. 2001). In algae, CAs were found in mitochondria, the chloroplast thylakoid, cytoplasm and periplasmic space (Moroney et al. 2001; Park et al. 1999; Badger and Price 1994). In C_3 dicotyledons two types of CAs have been isolated, one in the chloroplast stroma and one in cytoplasm, whereas in C_4 plants these enzymes are present in the mesophyll cells, where they provide bicarbonate to phosphoenolpyruvate (PEP) carboxylase, the first enzyme involved in fixation of CO_2 into C_4 acids (Badger and Price 1994). CAs are quite abundant in CAM (crassulacean acid metabolism) plants, being probably present in the cytosol and very abundant in chloroplasts, where they participate in CO_2 fixation, providing bicarbonate to PEP carboxylase (Badger and Price 1994). Plant CAs have been exhaustively reviewed by Badger and Price (1994). These enzymes are highly abundant in the terrestrial vegetation and seem to be correlated with the content of atmospheric CO_2 and thus with the global warming processes (Gillon and Yakir 2001).

In animals, and more specifically vertebrates, CAs are widespread. Because this field has recently been reviewed (Parkkila 2000; Supuran et al. 2003) and several chapters of this book deal with CAs present in diverse tissues of the human body (e.g., eyes, Chapter 8; tumor tissues, Chapter 9; gastrointestinal tract, Chapter 10; CNS, Chapter 10; kidneys, Chapter 10; skin, Chapter 11), the reader should consult these chapters for a detailed overview on the distribution and function of CAs.

1.4 PHYSIOLOGICAL FUNCTIONS OF CAs

It is not clear whether the other reactions catalyzed by CAs (Figure 1.1) except for CO_2 hydration/bicarbonate dehydration have physiological relevance (Supuran et al. 2003). Thus, at present, only the reaction in Equation 1.1, Figure 1.1, is considered to be the physiological one in which these enzymes are involved.

In prokaryotes, CAs possess two general functions: (1) transport of CO_2/bicarbonate between different tissues of the organism and (2) provide CO_2/bicarbonate for enzymatic reactions (Smith and Ferry 2000). In aquatic photosynthetic organisms, an additional role is that of a CO_2-concentrating mechanism, which helps overcome CO_2 limitation in the environment (Badger and Price 1994; Park et al. 1999). For example, in *Chlamydomonas reinhardtii*, this CO_2-concentrating mechanism is maintained by the pH gradient created across the chloroplast thylakoid membranes by Photosystem II-mediated electron transport processes (Park et al. 1999).

Many nonphotosynthesizing prokaryotes catalyze reactions for which CA are expected to provide CO_2/bicarbonate in the vicinity of the active site or to remove such compounds to improve the energetics of the reaction (Smith and Ferry 2000). Smith and Ferry (2000) have reviewed many carboxylation/decarboxylation processes in which prokaryotic CAs might play such an important physiological function.

In higher organisms, including vertebrates, the physiological functions of CAs have been widely investigated over the last 70 years (Maren 1967; Chegwidden and Carter 2000; Supuran et al. 2003). Thus, isozymes I, II and IV are involved in respiration and regulation of the acid/base homeostasis (Maren 1967; Chegwidden and Carter 2000; Supuran et al. 2003). These complex processes involve both the transport of CO_2/bicarbonate between metabolizing tissues and excretion sites (lungs, kidneys), facilitated CO_2 elimination in capillaries and pulmonary microvasculature, elimination of H^+ ions in the renal tubules and collecting ducts, as well as reabsorption of bicarbonate in the brush border and thick ascending Henle loop in kidneys (Maren 1967; Chegwidden and Carter 2000; Supuran et al. 2003). Usually, isozymes I, II and IV are involved in these processes. By producing the bicarbonate-rich aqueous humor secretion (mediated by ciliary processes isozymes CA II and CA IV) within the eye, CAs are involved in vision, and their misfunctioning leads to high intraocular pressure and glaucoma (Maren 1967; Supuran et al. 2003). CA II is also involved in bone development and function, such as differentiation of osteoclasts or providing acid for bone resorption in osteoclasts (Chegwidden and Carter 2000; Supuran et al. 2003). CAs are involved in the secretion of electrolytes in many other tissues and organs, such as CSF formation, by providing bicarbonate and regulating the pH in the choroid plexus (Maren 1967; Supuran et al. 2003); saliva production in acinar and ductal cells (Parkkila 2000); gastric acid production in the stomach parietal cells (Parkkila 2000; see also Chapter 10); and bile production, pancreatic juice production, intestinal ion transport (Parkkila 2000; Maren 1967; see also Chapter 10). CAs are also involved in gustation and olfaction, protecting the gastrointestinal tract from extreme pH conditions (too acidic or too basic), regulating pH and bicarbonate concentration in the seminal fluid, muscle functions and adapting to cellular stress (Chegwidden and Carter 2000; Parkkila 2000; Supuran et al. 2003). Some isozymes, e.g., CA V, are involved in molecular signaling processes, such as insulin secretion signaling in pancreas β cells (Parkkila 2000). Isozymes II and V are involved in important metabolic processes by providing bicarbonate for gluconeogenesis, fatty acids *de novo* biosynthesis or pyrimidine base synthesis (Chegwidden et al. 2000). Finally, some isozymes (e.g., CA IX, CA XII, CARP VIII) are highly abundant in tumors, being involved in oncogenesis and tumor progression (Pastorek et al. 1994; Chegwidden et al. 2001; Supuran et al. 2003; see also Chapter 9).

Although the physiological function of some isozymes (CA I, CA III, CARPs) is still unclear, the data presented here helps understand the importance of CAs in a host of physiological processes, both in normal and pathological states. This might explain why inhibitors of these enzymes found a place in clinical medicine by as early as 1954, with acetazolamide (**1.1**) being the first nonmercurial diuretic agent used clinically (Maren 1967). At present, inhibitors of these enzymes are widely used clinically as antiglaucoma agents, diuretics, antiepileptics, to manage mountain

sickness and for gastric and duodenal ulcers, neurological disorders, or osteoporosis. The development of more specific agents is required because of the high number of isozymes present in the human body as well as the isolation of many new representatives of CAs from all kingdoms. This is possible only by understanding in detail the catalytic and inhibition mechanisms of these enzymes. These enzymes and their inhibitors are indeed remarkable — after many years of intense research in this field, they continue to offer interesting opportunities to develop novel drugs and new diagnostic tools or to understanding in greater depth the fundamental processes of the life sciences.

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