Acta Cryst. (1994). D**50**, 731–738

Differences in Anionic Inhibition of Human Carbonic Anhydrase I Revealed from the Structures of Iodide and Gold Cyanide Inhibitor Complexes

BY VINAY KUMAR, K. K. KANNAN* AND P. SATHYAMURTHI

Solid State Physics Division, Bhabha Atomic Research Centre, Bombay-400085, *India*

(*Received* 22 *July* 1993; *accepted* 9 *February* 1994)

Abstract

The crystal structures of two anionic inhibitor complexes of human carbonic anhydrase I (HCAI), namely, HCAI–iodide and HCAI–$Au(CN)_2^-$, have been refined by the restrained least-squares method at 2.2 and 2 Å nominal resolution, respectively, with good stereochemistry for the final models. The *R* values have improved from 30.3 to 16.6% for HCAI–iodide and from 28.8 to 17.1% for HCAI–$Au(CN)_2^-$. The sites of inhibitor binding as elucidated are totally different in the two structures. The iodide anion replaces the zinc-bound H_2O/OH^- ligand and renders the enzyme inactive. This result confirms that the zinc-bound H_2O/OH^- is the activity-linked group in carbonic anhydrase enzymes. $Au(CN)_2^-$ binds at a different and new site near the zinc ion, without liganding to the metal. The N atom of $Au(CN)_2^-$ is within hydrogen-bonding distance of the zinc-bound H_2O/OH^- group which shifts by about 0.4 Å away from the zinc ion in relation to its position in the native HCAI. It is proposed that the presence of the inhibitor $Au(CN)_2^-$ results in a conformational reorientation of the activity-linked group, due to hydrogen-bond formation with the inhibitor, which in turn sterically hinders the binding of the substrate CO_2 molecule in the active site, leading to the inhibition of HCAI enzyme.

Introduction

Carbonic anhydrase (CA) enzymes (carbonate dehydratase, E.C. 4.2.1.1) are very efficient catalysts of the $CO_2 \rightarrow HCO_3^-$ interconversion reaction. Monovalent anions like I^-, $Au(CN)_2^-$ and SCN^-, inhibit the CO_2 hydration reaction in non-competitive/uncompetitive mode whereas the inhibition of the reverse reaction, *i.e.* dehydration of HCO_3^-, is best characterized as competitive (Pocker & Deits, 1982; Tibell, Forsman, Simonsson & Lindskog, 1984). A majority of the monovalent anions are expected to bind to the essential zinc ion in the active site, which is tetrahedrally liganded by three histidines and one water molecule in the native enzyme. Monovalent anions either displace the water molecule or bind at a different site, thus increasing the coordination number of the zinc ion (Bertini, Luchinat & Scozzafava, 1982; Hakansson, Carlsson, Svensson & Liljas, 1992; Lindahl, Svensson & Liljas, 1993).

The catalytic efficiency of CA enzymes, in particular that of the CAI and CAII forms, depends upon the basic form of an active-site group with a p*K* of about 7. On the basis of theoretical calculations and limited experimental evidence it has been generally accepted that the metal-bound H_2O, in the OH form, is the activity-linked group. The metal-bound imidazoles have also been considered as candidates for the same and this forms the basis of a few of the mechanistic proposals (Appleton & Sarkar, 1974). Even though I^- has been observed to displace the H_2O ligand on the metal, the exact binding site of the inhibitor I^- has not been elucidated unambiguously so far (Waara, Lovgren, Liljas, Kannan & Bergsten, 1972; Bertini *et al.*, 1982; Eriksson, Jones & Liljas, 1986).

The various mechanistic proposals also account for the formation of five-coordinated intermediate zinc species during catalysis (Kannan, Petef, Fridborg, Cid-Dresdner & Lovgren, 1977). It has been proposed from spectroscopic investigations of cobalt(II)-substituted bovine carbonic anhydrase (BCA)–$Au(CN)_2$ complex that the inhibitor binds directly to the metal ion (Bertini, Canti, Luchinat & Romanelli, 1980) by increasing its coordination to five. The two CN^- ligands of $Au(CN)_2$ could not be located unambiguously in electron-density maps in an earlier investigation (Bergsten *et al.*, 1971; Kannan, 1980*a*; Eriksson, Jones & Liljas, 1986). The $Au(CN)_2^-$ was bound at the active site, but its interaction with the enzyme has not been elucidated.

The zinc(II)-bound H_2O/OH^- had been shown to be involved in a conserved hydrogen-bond network in the active site and Kannan *et al.* (1977) and Merz (1990) had earlier assigned a definite functional role to this conserved OH^-–Thr199–Glu106 hydrogen-bonding network in CA enzymes. The presence of inhibitor anions in the active site is expected to perturb the hydrogen-bonding network.

* Author for correspondence.

The aim of the present study is to identify the activity-linked group, to understand the role of the active-site hydrogen-bond network and to elucidate the mechanism of inhibition by anionic inhibitors.

Experimental

Data collection and processing

Three-dimensional diffraction data, 92.8% complete to 2 Å resolution, collected earlier by the precession method for 40 layers (Kannan *et al.*, 1975), were used in the refinement of HCAI–$Au(CN)_2^-$.

Crystals of HCAI–iodide were obtained by soaking the native HCAI crystals in a solution of 2.5 *M* $(NH_4)_2SO_4$ and 0.05 *M* Tris–SO_4 (pH 8.5) containing 0.02 *M* NH_4I, for 24 h. The unit-cell parameters of the complex were found to be $a = 81.8$, $b = 75.2$, $c = 37.1$ Å and the space group was $P2_12_12_1$. The crystals were isomorphous with native HCAI and HCAI–$Au(CN)_2^-$ crystals.

Three-dimensional oscillation data from crystals of HCAI–I^-, mounted along a^* axis, were collected on CEA reflex films on an Arndt–Woncott oscillation camera using Ni-filtered Cu $K\alpha$ radiation from a GX-20 rotating-anode X-ray generator. 60 film packs covering 90° of reciprocal space were measured and processed on a Scandig-3 microdensitometer controlled by a PDP 11/34 computer, using an offline version of the *MOSCO* program package, modified from the original NOVA computer version kindly supplied by Dr A. J. Wonacott and implemented on the PDP11/34 system (Pal, Chakravarty & Kannan, 1984; Chakravarty & Kannan, 1990). The data were scaled using the 3*D* scaling program kindly provided by Dr D. Stuart and modified by us for a NORSK DATA 570 computer resulting in 10 061 unique reflections. The relevant statistics for this data set are given in Table 1.

Table 1. *Data-collection statistics for HCAI–iodide*

Resolution (Å)	Possible reflections	Observed reflections	% Completeness	Reflections with $F > 3\sigma_F$
∞–4.74	1341	1256	93.7	1151
4.74–2.99	3698	3502	94.6	2932
2.99–2.48	3597	3320	92.3	1798
2.48–2.37	1212	940	77.6	292
2.37–2.28	1177	767	65.2	165
2.28–2.20	1181	276	23.4	48
∞–2.20	12206	10061	82.4	6386

R_{merge} (on I) = 16.7%.

Table 2. *Progress of refinement*

At the end of each stage, the model was refitted against $(2F_o - F_c)$ and/or 'omit' Fourier maps.

Refinement stage No.	1	2	3	4	5	6	7
HCAI–$Au(CN)_2$							
No. of *PROLSQ* cycles	2	4	4	8	4	6	6
Total No. of atoms	2037	2106	2254	2229	2241	2266	2250†
No. of solvent and inhibitor atoms	1	70	218	193	205	230	243
$R_{initial}$ (%)	28.8	24.7	20.8	20.0	19.6	18.9	17.5
R_{final} (%)	26.3	22.6	19.9	19.3	18.3	17.7	17.1
	(For 14592 reflections with $I > 0$ in the 8–2 Å shell)						
HCAI–iodide							
No. of *PROLSQ* cycles	6	6*	6	10*	4	4	
Total No. of atoms	2234	2223	2223	2264	2308	2275†	
No. of solvent and inhibitor atoms	198	187	187	228	272	259	
$R_{initial}$ (%)	30.3	27.6	22.2	20.4	18.2	18.6	
R_{final} (%)	20.1	20.8	20.0	18.4	17.2	16.6	
	(For 9788 reflections with $I > 0$ in the 8–2.2 Å shell)						

* Individual *B* values of protein and solvent atoms were reset to their average values.

† The three amino-terminal residues in HCAI–I^- and the four amino-terminal residues in HCAI–$Au(CN)_2$, not observed in the electron-density maps, were dropped from the respective atomic models.

Refinement

The refined structure of HCAI (Kannan, Ramanadham & Jones, 1984) was used as the starting model in the refinement of both the structures. The position of I^- was located in difference Fourier maps, whereas the initial parameters for gold(I) were those reported by Kannan *et al.* (1975). The two structures were refined in stages (Table 2) by the stereochemically restrained least-squares method using the *PROLSQ* suite of programs (Hendrickson & Konnert, 1980) and manual model building on VG-3400 and IRIS-4D/20 graphics workstations using *FRODO/TOM* (Jones, 1982; Cambillau & Horjales, 1987) at the end of each stage of *PROLSQ* refinement. The improvement of the model was achieved by manually correcting the misplaced atoms and locating new solvent molecules using $(2F_o - F_c)$, $(F_o - F_c)$ and 'omit'-type maps (Vijayan, 1980; Bhat & Cohen, 1984). The Fourier maps were computed using the *PROTEIN* program package (Steigmann, 1974) modified for the NORSK DATA 570 computer. The scale factor and overall *B* value for each complex obtained from a Wilson plot were refined in the initial stages. In subsequent cycles of refinement, the scale factor and occupancy of newly added solvents and inhibitor anions, located from ΔF Fourier maps, were refined in addition to positional parameters and individual *B* values for all the atoms. Only solvent molecules with refined occupancy of more than 30% or *B* values of less than 58 $Å^2$ were retained in the model.

The weights used during the course of the refinement for various restraints and for the diffrac-

tion terms were similar to those suggested by Hendrickson (1985). However, after every cycle of model building the weight of the diffraction term was reduced in the subsequent two cycles of *PROLSQ* to improve the stereochemistry of the model. The stereochemistries of the final models were also evaluated by the *PROCHEK* suite of programs (Laskowski, MacArthur, Moss & Thornton, 1993).

Before cycles 7 and 23 of the refinement of HCAI–I^-, the individual *B* values of protein and solvent atoms were reset to their average values, as reported by Ramanadham, Sieker & Jensen (1990), in view of the limited quality of the data (Table 1).

The initial difference Fourier maps of HCAI–$Au(CN)_2^-$ clearly revealed the gold(I) position but only one of the CN^- groups pointing away from the zinc(II) ion. However, the other CN^- ligand could be located from subsequent difference Fourier maps during the course of the refinement. Initially only covalent distances (1–2 distances) were restrained. Angle restraints (1–3 distances) for $Au(CN)_2^-$ were used in the refinement after the fifth model building using computer graphics. Coordinate information from the crystal structure of $K[Au(CN)_2]$ (Rosenzweig & Cromer, 1959) was incorporated in the *PROLSQ* and *FRODO/TOM* restraint dictionaries.

Table 3. *Observed deviations of the refined models from ideal geometry*

Restraints	R.m.s. deviation from ideal value	
	HCAI–iodide	HCAI–$Au(CN)_2$
Distances (Å)		
Bond distances	0.016	0.017
Angle distances	0.036	0.036
Planar distances	0.048	0.047
Deviation from plane (Å)		
Planar groups	0.011	0.016
Chiral volume (Å^3)	0.169	0.183
Non-bonded contacts (Å)		
Single torsion	0.184	0.179
Multiple torsion	0.242	0.213
Possible hydrogen bonds	0.267	0.218
Torsion angles (°)		
Planar	5.7	3.1
Staggered	19.8	17.8
Orthonormal	30.3	31.0
Isotropic thermal factors (Å^2)		
Main-chain bond	2.0	2.3
Main-chain angle	2.8	3.3
Side-chain bond	2.7	3.7
Side-chain angle	3.6	5.2

Table 4. *Stereochemical parameters in HCAI–iodide and HCAI–*$Au(CN)_2^-$ *refined structures obtained using PROCHEK (Laskowski et al., 1993)*

	Parameter value	
	HCAI–iodide	HCAI–$Au(CN)_2^-$
Data resolution (Å)	2.2	2.0
% residues in the core φ, ψ region	84.3	90.1
Bad contacts/100 residues	1.2	0.8
χ_1 (*gauche* +) (°)	−63.5 ± 17.7	−64.8 ± 15.1
χ_1 (*gauche* −) (°)	+65.2 ± 14.6	+65.9 ± 11.0
χ_1 (*trans*) (°)	185.1 ± 15.1	183.4 ± 15.2
E.s.d. of pooled χ_1 (°)	16.3	14.0
φ (proline) (°)	−66.6 ± 11.6	−63.4 ± 12.8
Helix angle, φ (°)	−62.1 ± 9.3	−61.8 ± 13.5
Helix angle, φ (°)	−40.9 ± 11.0	−39.2 ± 8.2
Hydrogen-bond energy (kcal mol^{-1})	−1.9 ± 0.8	−2.0 ± 0.8
Trans ω (°)	179.5 ± 5.7	179.5 ± 3.1
C_α—N—C—C_β (°)	33.0 ± 3.2	33.3 ± 3.2

Results

The stereochemistry of the refined models is good as shown in Table 3 and the values of different stereochemical parameters are within the generally observed ranges as indicated in Table 4. The expected coordinate error estimated from Luzzati plots (Luzzati, 1952) is between 0.20 and 0.25 Å in HCAI–I^- and is 0.20 Å in HCAI–$Au(CN)_2^-$. Except for a few amino-terminal residues (see Table 2) and the side chains of Lys10, Arg173 and Glu221 (also that of Asp9 in HCAI–I^-), the quality of the electron-density maps was good in both the structures as can be seen from the representative regions given in Fig. 1.

Based on the high-resolution structure of HCAI–HCO_3^- (D_{min} = 1.6 Å) (Vinay Kumar & Kannan, unpublished results), residues 74 and 75 were corrected from the PDB entry (entry 2CAB, version of October 1984) (Kannan *et al.*, 1984) during the course of the refinement from Gln and Asp to Asp and Asn, respectively. These substitutions are also consistent with the chemical sequence of HCAI and the observed electron density in the Fourier maps of the two structures.

HCAI–iodide complex

The inhibitor I^- is found to replace the zinc(II)-coordinated H_2O/OH^- ligand. The corresponding difference electron density is shown in Fig. 2(*a*). The refined occupancy and the *B* value of this I^- are 77% and 14 Å^2, respectively, and the unrestrained Zn—I^- distance is 2.7 Å. Additional electron density (Fig. 2*b*) was observed in the vicinity of this I^-, which has been interpreted as due to two solvent molecules. In the initial phase of refinement, the SG atom of Cys212 shifted by about 2 Å into a heavy unaccounted peak in electron density, disturbing the stereochemistry around residue Cys212. This electron density was subsequently interpreted as corresponding to another I^- with a sulfur–iodide distance of 2.2 Å. Its occupancy and *B* value refined to 48% and 11.4 Å^2, respectively. Only a few stable sulfur–iodide bonds have so far been reported (Klapotze & Pass-

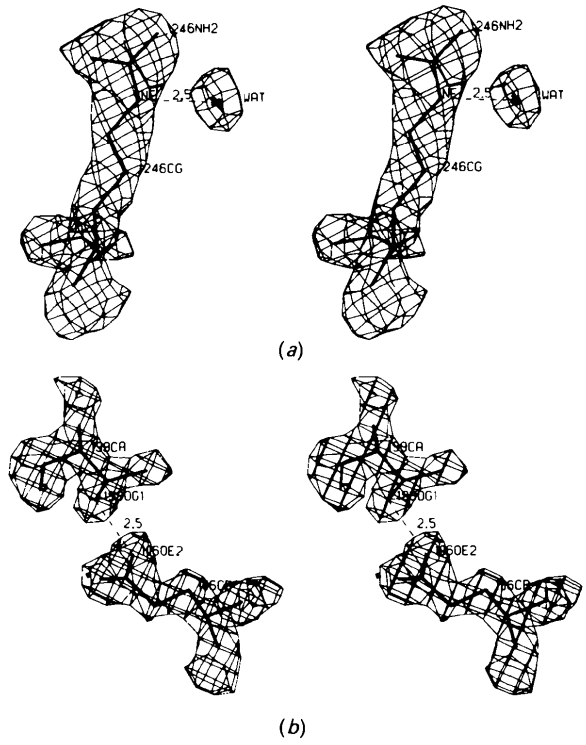

Fig. 1. The observed electron density in the final $(2F_o - F_c)$ map for (*a*) Arg246 and a solvent molecule in HCAI–I and (*b*) Glu106 and Thr199 in HCAI–Au$(CN)_2^-$. Contours are drawn at the $+1.5\sigma$ level.

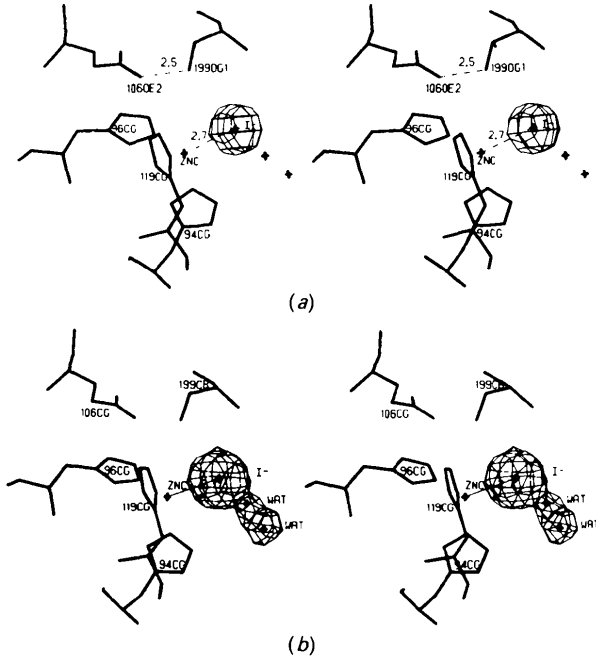

Fig. 2. Views of the $(F_o - F_c)$ 'omit' map corresponding to (*a*) I bound to the zinc ion in HCAI–iodide, with contours drawn at the $+10\sigma$ level and (*b*) I^- and two solvent molecules in its vicinity with contours drawn at the $+4\sigma$ level.

more, 1989) and this could be one such bond. However, the possibility that the peak is due to some metallic impurity, like a mercury(II) ion in the NH_4I used in the experiments, cannot be ruled out. During the later stages of refinement, three more solvent molecules associated with heavy electron density and negative *B* values were also treated as I^- ions. The occupancies and the *B* values of these sites refined to 38, 51 and 45%, and 25, 43 and 22 Å^2, respectively. These sites are in the vicinity of Glu14, Asn75 and Lys113, and no biological function can be assigned to them.

HCAI–Au$(CN)_2^-$ *complex*

$Au(CN)_2^-$, with a *B* value and an occupancy of 18.5 Å^2 and 51%, respectively, for the Au(I) ion, binds in the active site close to the zinc(II) ion with the nearest N atom at a distance of 3.4 Å from zinc(II) and a Zn–Au distance of 6.1 Å. The other *N* atom of $Au(CN)_2^-$ points into the opening of the active-site cavity and away from the zinc ion, and is at a distance of 9.2 Å from the zinc ion (Fig. 3). However, an abnormally low *B* value and a high occupancy for the distal CN^- and a heavy elongated residual electron density at this site indicated the presence of higher scattering matter. Another gold(I) ion was, therefore, included at this site partially overlapping with the C atom of the $Au(CN)_2^-$ (d = 0.5 Å). It refined to a *B* value and an occupancy of 12.6 Å^2 and 15%, respectively. This site is at a distance of 1.5 Å from the major gold(I) site.

Unlike I^-, $Au(CN)_2^-$ does not replace the zinc(II)-coordinated H_2O/OH^- ligand. An N atom of the $Au(CN)_2^-$ is within hydrogen-bonding distance (2.9 Å) of this H_2O/OH^- group. The most prominent effect of $Au(CN)_2^-$ binding is an increase in the Zn^{II}—OH^- bond length by 0.4 Å (Table 5). The stereochemistry of the active-site $Au(CN)_2^-$ in the

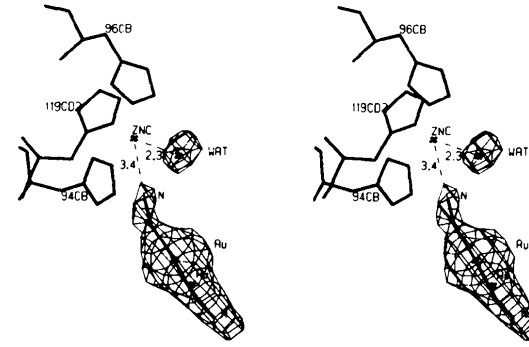

Fig. 3. Electron density in the $(F_o - F_c)$ 'omit' map for the active-site $Au(CN)_2$ anion and the activity-linked H_2O/OH^- group in HCAI–Au$(CN)_2^-$. Contours are drawn at the $+4\sigma$ level.

Table 5. *Comparative coordination geometry of the zinc ion in HCAI, HCAI–iodide and HCAI–*$Au(CN)_2^-$

Atom	Distance (Å) from OG1 of Thr199	Zinc ion	Angle (°)* X—Zn—94	X—Zn—96	X—Zn—119
HCAI					
$O(OH^-)$	2.9	1.9	98	125	112
His94 NE2		1.9		108	117
His96 NE2		1.9			98
His119 ND1		1.9			
HCAI–iodide					
I	3.6	2.7	105	123	103
His94 NE2		2.0		108	114
His96 NE2		1.9			105
His119 ND1		1.9			
HCAI–$Au(CN)_2$					
$O(OH^-)$	3.0	2.3	115	118	110
His94 NE2		1.9		104	111
His96 NE2		2.0			98
His119 ND1		2.0			
$N[Au(CN)_2^-]$		3.4			

* *X*, 94, 96 and 119 stands for the zinc ligands $O(OH^-)$/I, 94 NE2, 96 NE2 and 119 ND1, respectively.

Table 6. *Comparison of hydration and number of preserved solvent molecules in the native structure and the inhibitor complexes and solvent interactions with the main-chain and side-chain atoms in the three structures*

Solvent molecules displaced by less than 1 Å between two models are treated as preserved. Numbers in parentheses correspond to solvents interacting with the main-chain atoms. Also given is the number of solvent molecules which do not interact with protein atoms and form a secondary solvent shell.

(*a*) Hydration and number of preserved solvent molecules

Structure	Solvent molecules	HCAI–iodide	HCAI–$Au(CN)_2$
HCAI	195 (130)	83 (56)	124 (87)
HCAI–iodide	254 (94)	–	110 (54)
HCAI $Au(CN)_2$	233 (118)	–	–

(*b*) Solvent interactions

Structure	Main-chain Oxygen	Main-chain Nitrogen	Side-chain atoms	Secondary shell
HCAI	131	66	173	9
HCAI–iodide	106	35	130	91
HCAI–$Au(CN)_2$	124	53	141	32

complex is in good agreement with that found in the structure of $K[Au(CN)_2]$ (Rosenzweig & Cromer, 1959). However, the bond angle (N≡C—Au) involving the N atom pointing towards the zinc ion and hydrogen bonded with the H_2O/OH^- group, is distorted by 13° from the value of 172.8° in $K[Au(CN)_2]$. Two additional gold(I) sites, in the vicinity of Asn178 and Asn26, respectively, have also been identified. The site near Asn178 had also been reported by Kannan *et al.* (1975) from heavy-atom refinement during the course of multiple isomorphous replacement (MIR) structure determination of HCAI.

Comparison with the native structure

The r.m.s. displacement between the native HCAI structure (Kannan, 1990; Ramanadham & Kannan, unpublished results) and the structures of HCAI–I^- and HCAI–$Au(CN)_2^-$, and between the two inhibitor complexes, for all the equivalent protein atoms, are 0.42, 0.44 and 0.48 Å, respectively. Except for marginal changes in the side-chain orientations of Leu198 and His200 in HCAI–I^-, and in that of His200 in HCAI–$Au(CN)_2^-$, significant changes are not observed in the conformation of other active-site residues on inhibitor binding. However, variations are observed in the solvent structure of the three models (Table 6). The results presented in Table 6 conform to earlier reports that protein main-chain O atoms participate in more hydrogen bonds with the solvent molecules than main-chain N atoms do (Baker & Hubbard, 1984; Madhusudan, Kodandapani & Vijayan, 1993).

In spite of the variations in protein hydration, the prominent active-site hydrogen-bonding network, observed in native HCAI (Kannan *et al.*, 1984), is also maintained in the complex. This network, interconnecting active-site residues Thr199, Glu106, Tyr7, His64, His67 and His200, and a number of solvent molecules, has been conjectured to act as a proton-relay system (Kannan *et al.*, 1984).

Discussion

The structures of CA enzymes (Kannan *et al.*, 1984; Vinay Kumar, Kannan & Chidambaram, 1989; Hakansson *et al.*, 1992; Eriksson & Liljas, 1993) have revealed that the essential zinc(II), located at the apex of a funnel-shaped cavity, is approximately tetrahedrally liganded by three active-site residues His94, His96 and His119, and one water molecule. It is generally accepted that the zinc(II)-bound H_2O initiates the reaction and carries out the nucleophilic attack, in the OH^- form (Davis, 1959), on the electron-deficient carbon centre of the substrate CO_2 molecule.

In the structure of the HCAI–iodide complex, the inhibitor I^- replaces the fourth coordinated H_2O/OH^- ligand, without disturbing the distorted tetrahedral geometry around the zinc ion. The observed Zn–I^- distance of 2.7 Å in the complex structure agrees well with the distance of 2.65 Å reported from EXAFS studies on the BCA–iodide complex (Brown, Navon & Shulman, 1977). However, a Zn–I^- disance of 3.7 Å was reported from earlier investigations of HCAII–I^- based on heavy-atom refinement with MIR phases (Waara *et al.*,

1972). In contrast to results of this and related investigations of HCAII, which reported binding of I_3^- in the active site (Waara *et al.*, 1972 ; Eriksson *et al.*, 1986), we observe only one I^- in the active site of HCAI. The density corresponding to the two solvents in the vicinity of the active site I^- cannot, however, be interpreted to be oxidized iodide anions, as the observed I —OH_2—OH_2 angle in HCAI–I^- is 94° whereas I_3^- is a linear molecule. The refined *B* values of these solvent O atoms are 4.9 and 6.5 Å^2 with unit occupancy as compared to 14 Å^2 and an occupancy of 77% for the I^-. Also, the distances between these atoms in this cluster are 2.6 and 2.3 Å as compared to 2.83 and 3.03 Å observed in I_3^- (Cotton & Wilkinson, 1972).

Except for the substitution of zinc(II)-bound H_2O/OH^- with the inhibitor I^- and marginal positional shifts in Leu198 and His200 in the complex, the active-site structure is unperturbed when compared with that of the native HCAI. This result confirms earlier suggestions that the H_2O/OH^- ligand in the native enzyme is the activity-linked group (Davis, 1959; Silverman & Lindskog, 1988).

Unlike many other anionic inhibitors, $Au(CN)_2^-$ binds in a different part of the active-site cavity and is not bound directly to the zinc ion. The observed bending of the N≡C—Au angle, by 13° from the ideal value, facilitates the orientation of a lone pair on the N atom towards the positively charged zinc ion (Zn—N—C = 145°). The Zn—N distance of 3.4 Å may imply a weak interaction of the anion with the zinc, which may also explain the observed shift of 0.4 Å of the H_2O/OH^- group away from zinc, resulting from the partial neutralization of the positive charge on the zinc ion. However, from spectroscopic investigations of the cobalt(II)-substituted BCA—$Au(CN)_2^-$ complex it was postulated that the inhibitor anion binds to the zinc ion (Bertini *et al.*, 1980). Similar results have also been reported for other anionic inhibitor complexes like those of CN^- and CNO^- with HCAII (Lindahl *et al.*, 1993). It is indeed interesting that the binding site of $Au(CN)_2^-$ in HCAII (Eriksson, Jones & Liljas, 1986) is almost the same as that found in HCAI. The presence of the activity-linked zinc-coordinated solvent is also reported in the inhibitor complex of HCAII. In contrast to the results of the present studies, the CN^- groups of $Au(CN)_2^-$ could not be located in HCAIII–$Au(CN)_2^-$ and was, therefore, thought to be substituted by NH_3 from the crystallization media. Hence, the mechanism of inhibition of HCAII by $Au(CN)_2^-$ could not be clearly elucidated for the HCAII enzyme (Eriksson *et al.*, 1986).

In HCAI–$Au(CN)_2^-$ the electron-rich N atom closest to zinc(II) (Zn—N = 3.4 Å) is hydrogen bonded to the activity-linked group, with an O(H)—N distance of 2.9 Å. Model-building studies reveal an H(OH^-)–N[$Au(CN)_2^-$] distance of about 2 Å with an H—N—C angle of about 114°. Such interactions referred to as π-hydrogen bonds (Atwood, Hamada, Robinson, Orr & Vincent, 1991) have also been reported recently in other π-electron-rich systems (Sarma, Dhurjati, Bhanuprakash & Ravikumar, 1993; Viswamitra, Radhakrishnan, Bandekar & Desiraju, 1993). The formation of a hydrogen bond with the inhibitor N atom would hinder the zinc-bound OH^- in acting as donor to form a hydrogen bond to OG1 of the invariant Thr199 (Kannan *et al.*, 1984), considered important for the function of the enzyme (Kannan *et al.*, 1977; Merz, 1990). Instead, the H atom in the complex would be pointing towards the proposed substrate (CO_2) binding site which is in the vicinity of the zinc(II) ion (Kannan *et al.*, 1977), and in a pocket defined by His94, His119, Val121, Val143, Leu198, Thr199, Val207, Trp209 and the zinc(II)-bound OH^- group in HCAII (Liang & Lipscomb, 1990; Merz, 1991). This reorientation of the H atom would interfere with the substrate binding and thus inhibit the enzyme.

In the known crystal structures of CA enzymes, the zinc-bound H_2O/OH^- group is within hydrogen-bonding distance of the OG1 atom of Thr199 which in turn is hydrogen-bonded to an invariant Glu106. This hydrogen-bonding network has been considered important for catalysis by CA enzymes (Kannan *et al.*, 1977). Recently, using molecular-dynamics calculations, Merz (1990) proposed that the OH^- –Thr199–Glu106 network 'locks-in' the H atom of the OH^- in a position away from the possible substrate (CO_2) binding site, thus avoiding steric hindrance to substrate binding. This network also facilitates the nucleophilic attack of the oriented lone pair on the substrate molecule (Kannan, 1980*b*; Merz, 1990). This is in conformity with our proposal for $Au(CN)_2^-$ inhibition. From the crystallographic investigations of a number of anionic inhibitor complexes of HCAII, it has also been proposed that Thr199, as a hydrogen-bond acceptor, acts as a 'door keeper' and only protonated groups can occupy the H_2O/OH^- site, and unprotonated ligands bind at a different site on the zinc ion (Eriksson, Kylsten, Jones & Liljas, 1988; Xue *et al.*, 1993). This forms the basis of recent proposals on substrate HCO_3^- binding and the mechanism of action of the enzyme. I^- cannot, however, donate a proton, though it is found to replace the activity-linked H_2O/OH^- group in our studies and also in that of Eriksson *et al.* (1986). The same is also true of the SO_4^-, the Br^- and the N_3^- complexes of HCAII (Hakansson *et al.*, 1992; Jonsson, Hakansson & Liljas, 1993; Nair & Christianson, 1993). Interestingly, the binding site of Br^- is similar to that in our earlier proposal based on modelling studies (Vinay Kumar, 1992).

In HCAI–iodide, I^- is in van der Waals contact with the OG1 atom of Thr199 ($d = 3.6$ Å). Further, I^- is located in the pocket defined by the 198–200 loop of the protein (Fig. 4). Although the Zn—I distance of 2.7 Å in the complex is longer than the Zn—O(OH^-) distance of 1.9 Å in the native enzyme, the distorted tetrahedral coordination of the zinc(II) is preserved in the complex. The longer Zn—I^- distance results from the larger radius of I^-. The long $Au(CN)_2^-$ cannot bind in the same site as I^- due to steric hindrance from protein atoms of the 198–200 loop.

Concluding remarks

Our results confirm that the zinc(II)-bound H_2O/OH^- is the activity-linked group for CA enzymes. Replacement of this group, as observed in HCAI–I, results in the inhibition of CA enzymes. The conformational reorientation of the OH^- group, as in the case of HCAI–$Au(CN)_2^-$, could also abolish the catalytic activity. Our results show that OG1 of Thr199 is not a compulsive hydrogen-bond acceptor and unprotonated ligands can also replace the OH^- group. van der Waals interactions with OG1 of Thr199 and steric factors involving the Leu198 to His200 loop, in addition to hydrogen-bond interactions, are also important in accounting for the differences in the binding sites of various inhibitors of CA enzymes.

We are grateful to Dr R. Chidambaram, Dr M. Ramanadham and S. Chakravarty for many useful discussions. We thank Dr W. A. Hendrickson for *PROTEIN/PROLSQ*, Dr A. J. Wonacott for *MOSCO* film-processing software, Dr W. Steigmann for the *PROTEIN* package, Dr D. Stuart for *3D* scaling programs, Dr R. A. Laskowski for *PROCHECK* and Drs A. Jones and C. Cambillau for providing *FRODO/TOM*. Our sincere thanks are also due to the computer centre staff of BARC for providing excellent computing facilities. The refined coordinates for the complex structures and their observed structure factors have been deposited in the Brookhaven Protein Data Bank.*

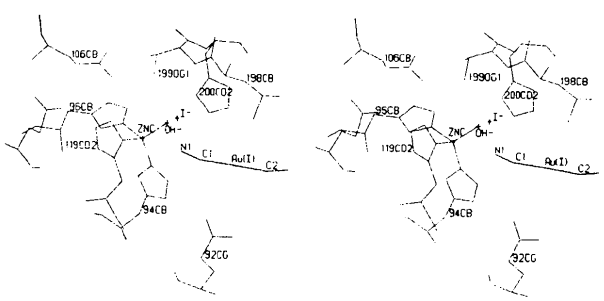

Fig. 4. The active site in HCAI enzyme. Also shown are binding sites of inhibitors I^- and $Au(CN)_2^-$.

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1HUG, 1HUH and R1HUGSF, R1HUHSF). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: VJ002).

References

APPLETON, D. W. & SARKAR, B. (1974). *Proc. Natl Acad. Sci. USA*, **71**, 1686–1690.

ATWOOD, J. L., HAMADA, F., ROBINSON, K. D., ORR, G. W. & VINCENT, R. L. (1991). *Nature (London)*, **349**, 683–684.

BAKER, E. N. & HUBBARD, R. E. (1984). *Progr. Biophys. Mol. Biol.* **44**, 97–179.

BERGSTEN, P. C., WAARA, I., LOVGREN, S., LILJAS, A., KANNAN, K. K. & BENGTSSON, U. (1971). *Proceedings of the Alfred Benzon Symposium* IV, Vol. VI, edited by C. M. RORTH & P. ASTRUP, pp. 363–383. Copenhagen: Munksgaard.

BERTINI, I., CANTI, G., LUCHINAT, C. & ROMANELLI, P. (1980). *Inorg. Chim. Acta*, **46**, 211–214.

BERTINI, I., LUCHINAT, C. & SCOZZAFAVA, A. (1982). *Struct. Bonding (Berlin)*, **48**, 45–92.

BHAT, T. N. & COHEN, G. H. (1984). *J. Appl. Cryst.* **17**, 244–248.

BROWN, G. S., NAVON, G. & SHULMAN, R. G. (1977). *Proc. Natl Acad. Sci. USA*, **74**, 1794–1797.

CAMBILLAU, C. & HORJALES, E. (1987). *J. Mol. Graph.* **5**, 174–177.

CHAKRAVARTY, S. & KANNAN, K. K. (1990). *J. Appl. Cryst.* **23**, 549–553.

COTTON, F. A. & WILKINSON, G. (1972). *Advanced Inorganic Chemistry*, 3rd ed. New York: Interscience.

DAVIS, R. P. (1959). *J. Am. Chem. Soc.* **81**, 5674–5678.

ERIKSSON, A. E., JONES, T. A. & LILJAS, A. (1986). *Zinc Enzymes*, edited by I. BERTINI, C. LUCHINAT, W. MARET & M. ZEPPEZAUER, pp. 317–328. Boston: Birhauser.

ERIKSSON, A. E., KYLSTEN, P. M., JONES, T. A. & LILJAS, A. (1988). *Proteins Struct. Funct. Genet.* **4**, 283–293.

ERIKSSON, A. E. & LILJAS, A. (1993). *Proteins Struct. Funct. Genet.* **16**, 29–42.

HAKANSSON, K., CARLSSON, M., SVENSSON, A. L. & LILJAS, A. (1992). *J. Mol. Biol.* **227**, 1192–1204.

HENDRICKSON, W. A. (1985). *Methods in Enzymology*, Vol. 115, edited by H. W. WYCKOFF & C. H. W. HIRS, pp. 252–270. New York: Academic Press.

HENDRICKSON, W. A. & KONNERT, J. H. (1980). *Computing in Crystallography*, edited by R. DIAMOND, S. RAMASESHAN & K. VENKATESAN, pp. 13.01–13.23. Bangalore: Indian Academy of Sciences.

JONES, T. A. (1982). *Computational Crystallography*, edited by D. SAYER, pp. 303–317. Oxford: Clarendon Press.

JONSSON, B. M., HAKANSSON, K. & LILJAS, A. (1993). *FEBS Lett.* **322**, 186–190.

KANNAN, K. K. (1980*a*). *Biomolecular Structure, Conformation, Function and Evolution*, edited by R. SRINIVASAN, pp. 165–181. Oxford: Pergamon Press.

KANNAN, K. K. (1980*b*). *Biophysics and Physiology of Carbon Dioxide*, edited by C. BANNER, G. GROS & H. BARTELS, pp. 165–176. Berlin: Springer-Verlag.

KANNAN, K. K. (1990). *Photon Fact. Act. Rep. Jpn*, **8**, 94.

KANNAN, K. K., NOSTRAND, B., FRIDBORG, K., LOVGREN, S., OHLSSON, A. & PETEF, M. (1975). *Proc. Natl Acad. Sci. USA*, **72**, 51–55.

KANNAN, K. K., PETEF, M., FRIDBORG, K., CID-DRESDNER, D. & LOVGREN, S. (1977). *FEBS Lett.* **73**, 115–119.

KANNAN, K. K., RAMANADHAM, M. & JONES, T. A. (1984). *Ann. N. Y. Acad. Sci.* **429**, 49–60.

KLAPOTZE, T. & PASSMORE, J. (1989). *Acc. Chem. Res.* **22**, 234–240.

LASKOWSKI, R. A., MACARTHUR, M. W., MOSS, D. S. & THORNTON, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.

LIANG, J. U. & LIPSCOMB, W. N. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 3675–3679.

LINDAHL, M., SVENSSON, L. A. & LILJAS, A. (1993). *Proteins Struct. Funct. Genet.* **15**, 177–182.

LUZZATI, V. (1952). *Acta Cryst.* **5**, 802–810.

MADHUSUDAN, KODANDAPANI, R. & VIJAYAN, M. (1993). *Acta Cryst.* D**49**, 234–245.

MERZ, K. M. (1990). *J. Mol. Biol.* **214**, 799–802.

MERZ, K. M. (1991). *J. Am. Chem. Soc.* **113**, 406–411.

NAIR, C. K. & CHRISTIANSON, D. W. (1993). *Eur. J. Biochem.* **213**, 507–515.

PAL, P. K., CHAKRAVARTY, S. & KANNAN, K. K. (1984). *Methods in Crystallographic Computing*, edited by S. R. HALL & T. ASHIDA, p. 475. Oxford: Clarendon Press.

POCKER, Y. & DEITS, T. L. (1982). *J. Am. Chem. Soc.* **104**, 2424–2434.

RAMANADHAM, M., SIEKER, L. C. & JENSEN, L. H. (1990). *Acta Cryst.* B**46**, 63–69.

ROSENZWEIG, A. & CROMER, D. T. (1959). *Acta Cryst.* **12**, 709–712.

SARMA, J. A. R., DHURJATI, M. S. K., BHANUPRAKASH, K. & RAVIKUMAR, K. (1993). *J. Chem. Soc. Chem. Commun.* **5**, 440–442.

SILVERMAN, D. N. & LINDSKOG, S. (1988). *Acc. Chem. Res.* **21**, 30–36.

STEIGMANN, W. (1974). PhD thesis. Technische Univ. Munich, Germany.

TIBELL, L., FORSMAN, C., SIMONSSON, I. & LINDSKOG, S. (1984). *Biochem. Biophys. Acta*, **789**, 302–310.

VIJAYAN, M. (1980). *Acta Cryst.* A**36**, 295–298.

VINAY KUMAR, (1992). PhD thesis. Univ. of Bombay, Bombay, India.

VINAY KUMAR, KANNAN, K. K. & CHIDAMBARAM, R. (1989). *Curr. Sci.* **58**, 344–348.

VISWAMITRA, M. A., RADHAKRISHNAN, R., BANDEKAR, J. & DESIRAJU, G. R. (1993). *J. Am. Chem. Soc.* **115**, 4868–4869.

WAARA, I., LOVGREN, S., LILJAS, A., KANNAN, K. K. & BERGSTEN, P. C. (1972). *Proceedings on Hemoglobin and Red Cell Structure and Function*, edited by G. J. BREWER, pp. 169–187. New York: Plenum Press.

XUE, Y., VIDGREN, J., SVENSSON, L. A., LILJAS, A., JONSON, B. H. & LINDSKOG, S. (1993). *Proteins Struct. Funct. Genet.* **15**, 80–87.