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X-ray Analysis of Metal-Substituted Human Carbonic Anhydrase II Derivatives

BY KJELL HÅKANSSON

Molecular Biophysics, Chemical Center, University of Lund, PO Box 124, *S*-221 00 *Lund, Sweden*

ALLAN WEHNERT

Janssenpharma A/S, PO Box 149, *Hammerbakken* 19, *DK*-3460 *Birkerød, Denmark*

AND ANDERS LILJAS

Molecular Biophysics, Chemical Center, University of Lund, PO Box 124, *S*-221 00 *Lund, Sweden*

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Abstract

Metal-substituted crystals of human carbonic anhydrase II belonging to space group *P*2/1 with cell dimensions a = 42.7, b = 41.7, c = 73.0 Å and β = 104.6° were analyzed crystallographically. The resolution limit ranged from 1.82 to 1.92 Å with high completeness (86.2-90.7%). Cobalt(II)-substituted carbonic anhydrase has a tetrahedral coordination around the metal both at pH 6 and pH 7.8, similar to the native zinc enzyme. In contrast, the catalytically inactive copper(II), nickel(II) and manganese(II) derivatives showed increased coordination number around the metal ion. Whereas the copper is best described as penta-coordinated, the nickel and manganese are best described as hexa-coordinated. The results are briefly compared with spectroscopic observations and our current view on carbonic anhydrase catalysis.

1. Introduction

Human carbonic anhydrase II is an erythrocytic zinc enzyme that catalyzes the interconversion of carbon dioxide and bicarbonate. The zinc ion can be removed by chelators and replaced by a wide variety of other metal ions (Hunt, Rhee & Storm, 1977). Cobalt(II) carbonic anhydrase has 50% of the carbon dioxide hydration activity of the native zinc enzyme. In contrast, the activities of the cupric, ferrous, nickel(II) and manganese(II) derivatives are negligible (Coleman, 1967; Lindskog & Nyman, 1964; Thorslund & Lindskog, 1967), despite the similarity of these metal ions to zinc in terms of charge, size and pK. The three-dimensional structure of human carbonic anhydrase II is known at high resolution (Liljas, Kannan, Bergstén, Waara, Fridborg, Strandberg, Carlbom, Järup, Lövgren & Petef, 1972; Eriksson, Jones & Liljas, 1988; Håkansson, Carlsson, Svensson & Liljas, 1992). The zinc ion, which is tetrahedrally coordinated by three histidine residues and a water molecule, is located in a cavity which is bordered by a hydrophobic β-sheet that runs through the molecule. Carbon dioxide hydration results from nucleophilic attack by the zinc-bound water. The structure of the active-site cavity after metal substitution was investigated in order to explain the different degrees of activity displayed by the metal-substituted derivatives.

2. Materials and methods

2.1. *Preparation of crystals*

Human carbonic anhydrase II was crystallized in 2.4 *M* ammonium sulfate, 50 m*M* Tris-HCl pH 8.5 and 1 m*M* $HgCl_2$ (Tilander, Strandberg & Fridborg, 1965). Removal of mercury was achieved by soaking the crystals in 3 *M* ammonium sulfate, 50 m*M* Tris pH 7.8 and 5 m*M* β-mercaptoethanol for 1-2 d with 3-4 changes of solvent.

Apo-enzyme crystals were prepared as described (Hunt, Rhee & Storm, 1977; Håkansson, Carlsson, Svensson & Liljas, 1992). The metal derivatives were prepared by soaking the apo-enzyme crystals in 3 *M* ammonium sulfate, 50 m*M* Tris pH 7.8 and 2 m*M* $CoCl_2$, $MnSO_4$, $CuSO_4$ or $NiCl_2$ for 2 d. The oxidation state of the cobalt ion survives the treatment as has been confirmed by Håkansson & Wehnert (1992).

2.2. *Data collection and structure refinement*

X-ray diffraction data were collected and processed as described (Håkansson, Carlsson, Svensson & Liljas, 1992). The number of reflections, resolution, completeness and R_{merge} values are shown in Table 1.

Restrained least-squares refinement (Hendrickson, 1985) was performed using the program *PROFFT* (Finzel, 1987) with the native enzyme as starting model (Håkansson, Carlsson, Svensson & Liljas, 1992). Plots were prepared by the program *ESVodo* (Pflugrath, Saper & Quiocho, 1984). Coordinate root-mean-square deviations were estimated from a Luzzati plot (Luzzati, 1952). Refinement statistics are shown in Table 2. Coordinates and F values have been deposited with

Table 1. *Data-collection summary*

$R_m = \sum_{hkl}\sum_j(\langle I(hkl)\rangle - I(hkl)j)/\sum_{hkl}\langle I(hkl)\rangle$.

Shell lower angle limit	No. of scaled observations	No. of unique reflections	Bragg possible	Completness to lower limit (%)	Average intensities	Average $I/\sigma(I)$	R_m	Detector swing (°)
Cobalt(II) enzyme pH 7.8								
3.41	13354	3512	3525	99.6	1190	79.7	5.4	
2.71	11467	3407	3414	99.7	500	35.9	6.4	
2.37	9844	3400	3418	99.6	264	18.6	8.8	
2.15	8681	3334	3407	99.2	207	13.3	10.3	
1.99	7005	3194	3388	98.2	160	8.7	12.6	
1.88	2686	1694	3409	90.2	124	5.5	14.7	
Totals	53037	18541	20561	90.2	442	29.5	5.9	18
Cobalt(II) enzyme pH 6.0								
3.30	13616	3884	3898	99.6	1176	73.8	5.5	
2.62	11906	3805	3823	99.6	448	30.2	6.9	
2.29	10058	3757	3805	99.3	257	16.0	9.2	
2.08	8810	3678	3762	98.9	205	11.2	11.2	
1.93	7131	3626	3806	98.2	146	6.8	14.4	
1.82	3078	1973	3759	90.7	109	4.4	18.0	
Totals	54599	20723	22853	90.7	422	25.9	6.0	20
Copper(II) enzyme								
3.49	13342	3267	3289	99.3	392	49.7	6.3	
2.77	10903	3202	3220	99.4	185	19.1	9.7	
2.42	9034	3101	3205	98.5	97	8.8	14.8	
2.20	7767	2945	3163	97.2	75	6.1	17.9	
2.04	6192	2699	3177	94.8	59	4.1	21.8	
1.92	2410	1378	3190	86.2	51	2.6	25.6	
Totals	49648	16592	19244	86.2	158	17.1	7.9	17
Nickel(II) enzyme								
3.49	13542	3229	3318	97.3	544	57.5	6.1	
2.77	11056	3229	3243	98.4	257	23.6	8.5	
2.42	9348	3196	3226	98.6	137	11.4	12.3	
2.20	8253	3170	3221	98.6	108	8.1	14.4	
2.04	6369	3051	3213	97.9	87	5.4	16.6	
1.92	2426	1593	3193	90.0	70	3.6	19.4	
Totals	50994	17468	19414	90.0	215	20.0	7.2	17
Manganese(II) enzyme								
3.41	12910	3495	3509	99.6	1224	78.6	5.4	
2.71	11186	3399	3412	99.6	510	34.8	6.4	
2.37	9588	3311	3406	98.8	268	18.0	9.0	
2.15	8378	3133	3378	97.3	208	12.9	10.9	
2.00	6781	2872	3374	94.9	162	8.9	13.3	
1.88	2954	1662	3402	87.3	120	5.4	16.2	
Totals	51797	17872	20481	87.3	460	29.5	5.8	18

Table 2. *Refinement statistics*

$R_{cryst} = \sum(|F_o| - |F_c|)/\sum|F_o|$.

	Target σ	Cobalt(II) derivative (pH 7.8)	Cobalt(II) derivative (pH 6)	Copper(II) derivative	Nickel(II) derivative	Manganese(II) derivative
R_{cryst}		0.151	0.155	0.150	0.150	0.148
Mean B (Å^2)		14.5	14.7	15.1	13.9	15.2
Error in coordinates (Å) according to Luzzati plot	0.16	0.16	0.17	0.16	0.16	
R.m.s. values						
Distances						
Bond distance (Å)	0.020	0.020	0.019	0.020	0.020	0.021
Angle distance (Å)	0.030	0.038	0.036	0.038	0.037	0.040
Planar 1–4 distance (Å)	0.050	0.054	0.046	0.049	0.047	0.053
Miscellaneous						
Plane groups (Å)	0.020	0.018	0.018	0.019	0.018	0.019
Chiral centers (Å^3)	0.150	0.228	0.224	0.231	0.219	0.239
Non-bonded distances						
Single torsion (Å)	0.200	0.166	0.157	0.158	0.159	0.160
Multiple torsion (Å)	0.200	0.158	0.159	0.165	0.158	0.156
Possible X—Y hydrogen bond (Å)	0.200	0.162	0.152	0.153	0.159	0.154
Torsion angles						
Planar (°)	3.0	3.5	3.4	3.4	3.4	3.5
Staggered (°)	15.0	15.9	16.0	17.2	16.5	15.7
Orthonormal (°)	20.0	30.6	30.7	30.9	30.7	30.4
Thermal restraints						
Main-chain bond (Å^2)	1.000	1.022	1.007	0.968	0.976	1.038
Main-chain angle (Å^2)	1.500	1.637	1.623	1.567	1.575	1.658
Side-chain bond (Å^2)	1.500	2.193	2.055	1.962	2.002	2.286
Side-chain angle (Å^2)	2.000	3.403	3.235	3.039	3.129	3.504

the Protein Data Bank* (Bernstein, Koetzle, Williams, Meyer, Brice, Rodgers, Kennard, Shimanouchi & Tasumi, 1982).

3. Results

3.1. *Native zinc enzyme*

The structure of the active site in the zinc enzyme (Håkansson, Carlsson, Svensson & Liljas, 1992) is shown in Fig. 1 for comparison.

3.2. *Cobalt*(II) *enzyme at pH* 7.8

The tetrahedral geometry around the active-site metal in native carbonic anhydrase is preserved in the cobalt(II)-substituted derivative (Table 3). The distance between the metal-bound waters in native and cobalt-substituted carbonic anhydrase is only 0.1 Å. The only significant difference between the two enzymes is that the deep water (338) is absent and that a new ligand appears on this side of the zinc water. This molecule has not been unambiguously defined, but molecular oxygen was successfully modelled into its density. In contrast, neither water nor carbon dioxide could be fitted into the maps. The distance from the O atom to the Thr199 N atom is 3.5 Å. The other end of it is 2.7 Å from the cobalt

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1RZA, 1RZB, 1RZC, 1RZD, 1RZE, R1RZASF, R1RZBSF, R1RZCSF, R1RZDSF, R1RZESF). Free copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Supplementary Publication No. SUP 37098). A list of deposited data is given at the end of this issue.

Table 3. *Geometries in the active site of cobalt*(II) *carbonic anhydrase* II *at pH* 7.8

Distances (X—Co) and angles (X—Co—Y) of the cobalt ion ligands.

	Distances (Å)	Angles (°)			
		His94 Nε2	His96 Nε2	His119 Nδ1	O500 O2
Wat263	2.06	105.8	104.8	129.0	58.4
His94 Nε2	2.16		105.4	112.6	93.0
His96 Nε2	2.15			96.2	158.5
His119 Nδ1	2.12				86.8
O500 O2	2.72				

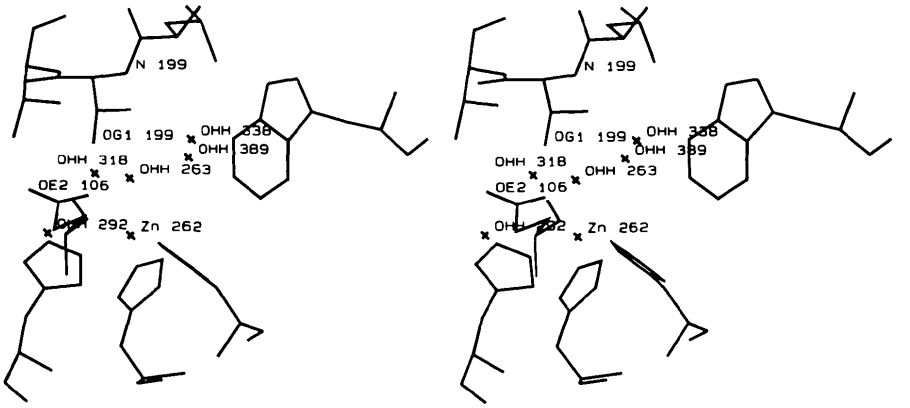

Fig. 1. The active-site structure of native human carbonic anhydrase II. The conformation of the peptide chain is the same in the native enzyme and all the metal-substituted derivatives described in this paper, but the solvent structure differs among the different substitutions. Waters 263 and 338 are sometimes referred to as the 'zinc water' and 'deep water', respectively. The structure was plotted using the coordinates of Håkansson, Carlsson, Svensson & Liljas (1992).

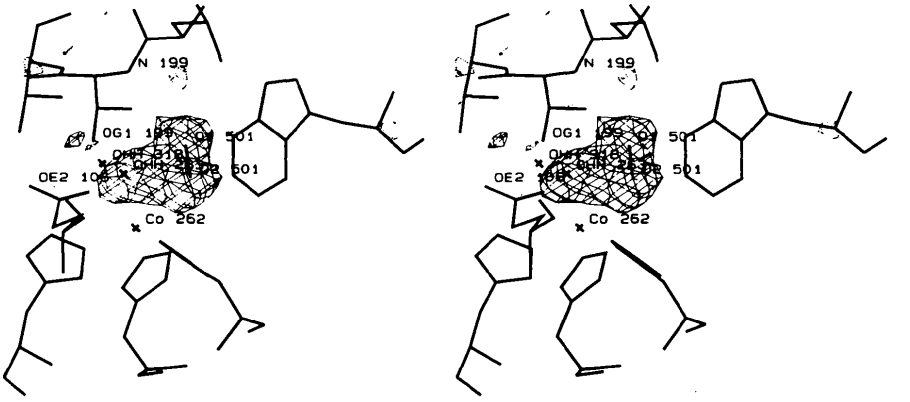

Fig. 2. The active site of cobalt(II)-substituted carbonic anhydrase at pH 7.8. Difference electron maps were calculated after refinement of native coordinates without waters 263 and 338. Positive (continuous lines) and negative (broken lines) $|F_o| - |F_c|$ contours were drawn at $+3\sigma$. An oxygen molecule has been fitted to the positive density close to the zinc ion.

Table 4. *Geometries in the active site of cobalt*(II) *carbonic anhydrase* II *at pH* 6

Distances (X—Co) and angles (X—Co—Y) of the cobalt ion ligands.

	Distances (Å)	Angles (°)			
		His94 Nε2	His96 Nε2	His119 Nδ1	Sul500 O2
Sul500 O3	2.16	109.2	97.0	119.8	62.1
His94 Nε2	2.17		105.0	116.2	82.8
His96 Nε2	2.14			99.3	166.9
His119 Nδ1	2.15				86.3
Sul500 O2	2.62				

ion. Both ends are 2.4 Å from the zinc water, which in turn is 2.9 Å away from Thr199 Oγ1. The situation is illustrated in Fig. 2.

3.3. *Cobalt*(II) *enzyme at pH* 6.0

At low pH, a sulfate ion (Sul500) replaces the metal bound water and the deep water. The occupancy was refined to 0.6. Two of its O atoms utilize the previously observed carboxylate-binding site (Håkansson & Wehnert, 1992) with O1 2.9 Å from Thr199 N and O2 2.6 Å from the cobalt ion. Sul500 O3 is bound 0.3 Å from the native zinc water position and Sul500 O4 points out towards the active-site entrance. The binding is rather similar to sulfonamide binding to native carbonic anhydrase, but the metal-Sul500 O2 distance is smaller than the corresponding distance in these complexes (Vidgren, Svensson & Liljas, 1993). Waters 263, 338, 381 and 389 are absent, the position of the latter in native carbonic anhydrase is 1.6 Å from Sul500 O4. The Sul500 O3 is tetrahedrally bonded to Sul500 S, Thr199 Oγ1, the cobalt ion and water 318. The complex is shown in Fig. 3, and the distances and angles around the cobalt ion are listed in Table 4.

3.4. *Copper*(II) *enzyme*

The active site in (inactive) copper(II)-substituted carbonic anhydrase II is shown in Fig. 4. As in the cobalt(II) derivative, an unidentified molecule has replaced the deep water, and again molecular oxygen was fitted into the maps. One of its atoms is 3.5 Å away from the amide N atom of Thr199, and the other only 2.4 Å from the metal. The zinc water has moved 1.2 Å from its native

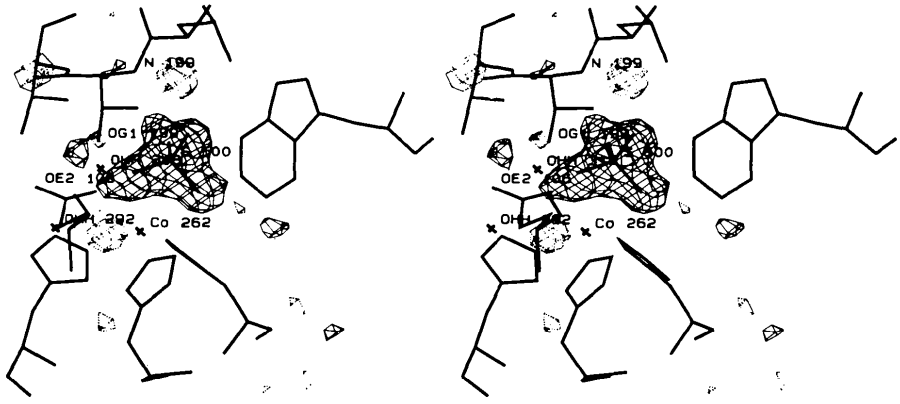

Fig. 3. The active site of cobalt(II)-substituted carbonic anhydrase at pH 6.0. Difference electron maps were calculated after refinement of native coordinates without waters 263, 338 and 339. Positive (continuous lines) and negative (broken lines) $|F_o| - |F_c|$ contours were drawn at $+3\sigma$. The occupancy of the sulfate was refined to 0.6.

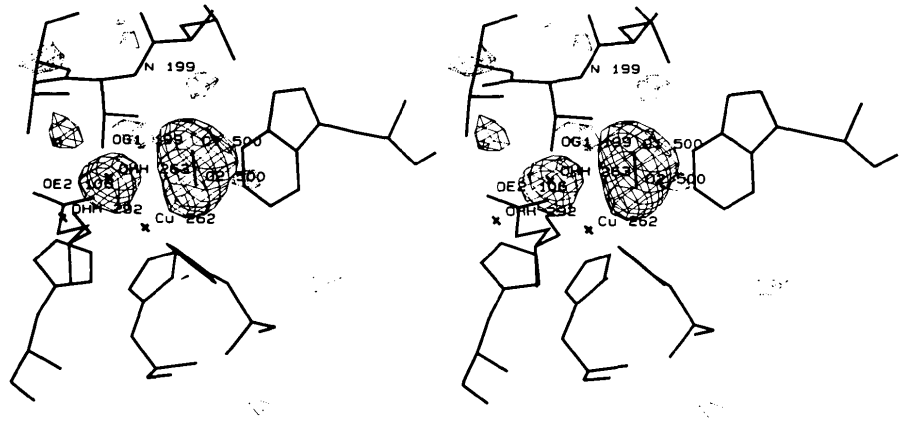

Fig. 4. The 'active site' of copper(II)-substituted carbonic anhydrase at pH 7.8. Difference electron maps were calculated after refinement of native coordinates without waters 263, 318 and 338. Positive (continuous lines) and negative (broken lines) $|F_o| - |F_c|$ contours were drawn at $+3\sigma$. An oxygen molecule has been fitted to the positive density close to the zinc ion.

Table 5. *Geometries in the active site of copper(II) carbonic anhydrase* II

Distances (X—Cu) and angles (X—Cu—Y) of the copper ion ligands.

	Distances (Å)	Angles (°) His94 Nε2	His96 Nε2	His119 Nδ1	O500 O2
Wat263	2.26	100.0	83.8	141.6	82.6
His94 Nε2	2.30		92.9	118.3	100.0
His96 Nε2	2.21			95.6	164.4
His119 Nε2	2.12				90.3
O500 O2	2.38				

position and is 2.8 Å from Thr199 Oγ1. The atoms of the oxygen molecule are both 3.1 Å away from the metal-bound water. Their distances to the native zinc water position are 2.0 and 1.9 Å, respectively. Both these atoms are 0.2 Å closer to the hydrophilic side of the active site than they are in the cobalt(II)-substituted derivative. The distances and angles of the copper(II) ligands are shown in Table 5. Water 318 is absent. Since the native zinc water position is not utilized, and since we have two non-peptide ligands within 2.4 Å, the geometry is best described as penta-coordinated, with four ligands essentially in one plane and His94 in an apical position.

Fig. 5 shows a second bound copper ion, complexed to the less abundant conformation of His64 and one of the His4 conformations. The distance from the copper ion to the closest (ε) atom of His64 is 2.6 Å. The corresponding value to His4 is 2.5 Å. The occupancy of this copper ion was refined to 0.2, the same value as for the less abundant conformation of His64.

Structure determination of the copper(II)-substituted enzyme in the presence of 10 mM ammonium bicarbonate yielded the same result (data not shown); a diatomic molecule rather than bicarbonate was observed in the active site.

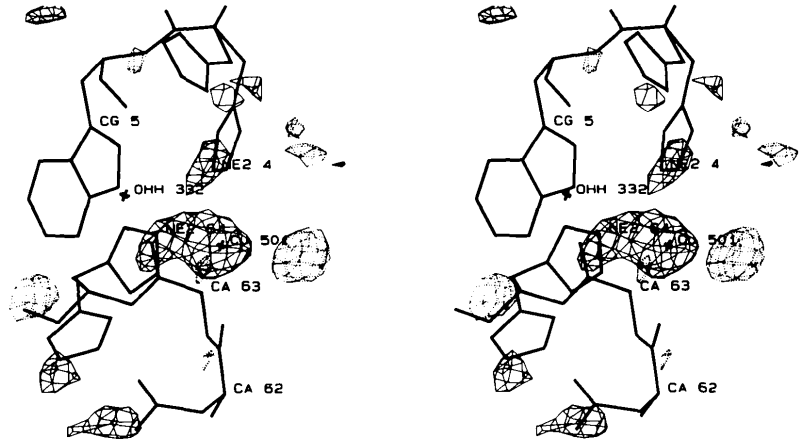

Fig. 5. The second copper site in copper(II)-substituted carbonic anhydrase at pH 7.8. Difference electron maps were calculated after refinement of native coordinates (without waters 263, 318 and 338). Positive (continuous lines) and negative (broken lines) $|F_o| - |F_c|$ contours were drawn at $+3\sigma$. The occupancy of this copper was refined to 0.2, the same value as for the His64 conformation to which it is bound. This binding of copper to His64 is probably related to the inhibition of proton transfer from and to the active site (Tu, Wynns & Silverman, 1981). The position is different from the binding sites for mercury to His64 (Eriksson, Kylsten, Jones & Liljas, 1988).

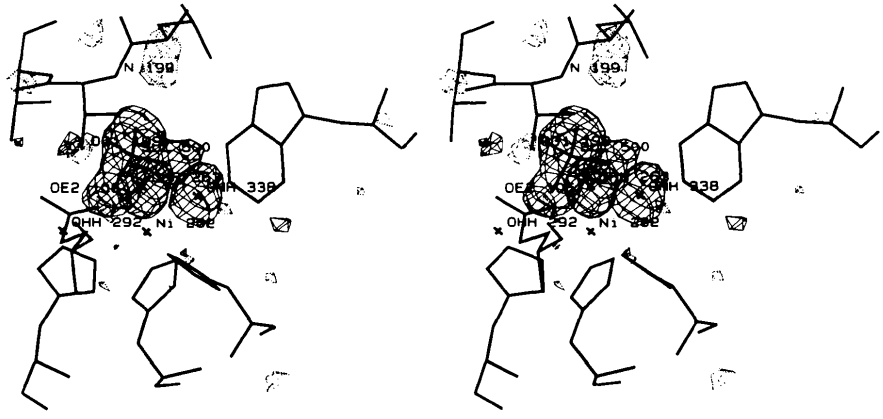

Fig. 6. The 'active site' of nickel(II)-substituted carbonic anhydrase at pH 7.8. Difference electron maps were calculated after refinement of native coordinates without waters 263, 318 and 338. Positive (continuous lines) and negative (broken lines) $|F_o| - |F_c|$ contours were drawn at $+3\sigma$. The occupancy of the sulfate was refined to 0.7.

Table 6. *Geometries in the active site of nickel*(II) *carbonic anhydrase* II

Distances (X—Ni) and angles (X—Ni—Y) of the nickel ion ligands.

	Distances (Å)	Angles (°) Wat338	His94 Nε2	His96 Nδ2	His119 Nδ1	Sul500 O1
Wat263	2.16	77.0	167.6	94.0	83.9	81.9
Wat338	2.33		95.4	170.8	88.6	87.3
His94 Nε2	2.21			93.8	105.9	88.1
His96 Nε2	2.26				88.6	93.3
His119 Nδ1	2.20					165.7
Sul500 O1	2.33					

Table 7. *Geometries in the active site of manganese*(II) *carbonic anhydrase* II

Distances (Mn—X) and angles (X—Mn—Y) of the manganese ion ligands.

	Distances (Å)	Angles (°) His94 Nε2	His96 Nε2	His119 Nδ1	Sul500 O2	Sul500 O3
Wat263	2.31	88.7	81.5	159.5	90.8	74.7
His94 Nε2	2.28		92.2	111.8	88.6	141.4
His96 Nε2	2.34			96.0	172.3	118.5
His119 Nδ1	2.15				90.8	89.0
Sul500 O2	2.56					57.8
Sul500 O3	2.43					

3.5. *Nickel*(II) *enzyme*

In a similar manner to the cobalt derivative at low pH, the nickel derivative also contains a sulfate with partial occupancy (0.7), but the position of the sulfate is very different. The complex is shown in Fig. 6. Two water molecules and a sulfate ion are within 2.3 Å from the metal ion, and the angles, shown in Table 6, are typical for hexa-coordination (close to 90 or 180°). The water molecules are labelled 263 and 338, but this is rather arbitrary, since their positions differ from the native 263 and 338 positions. The distances between the native zinc water position and Sul500 O1, water 263 and water 338 of the nickel enzyme are 1.3, 1.7 and 2.4 Å, respectively. The sulfate is located more towards the hydrophilic side of the active site compared with the cobalt enzyme at low pH, but one of its O atoms is still hydrogen bonded to Thr199 N (2.6 Å), and the metal-coordinated O atom is 2.5 Å from Thr 200 Oγ1. Water 263 is 2.6 Å from Thr199 Oγ1, but no atom of the sulfate is within hydrogen-bonding distance of this atom.

3.6. *Manganese*(II) *enzyme*

Electron density at the hydrophobic side of the metal position was again interpreted as a sulfate ion, but this time with an occupancy of only 0.4. Since the structure of the free manganese enzyme remains unknown, the results must be interpreted with caution. Water 263 and sulfate O atoms O2 and O3 are all within 2.6 Å from the metal and the situation resembles the complex between cobalt-substituted carbonic anhydrase and bicarbonate (Håkansson & Wehnert, 1992). The coordination geometry is best described as roughly octahedral and is shown in Table 7 and Fig. 7. No atom is closer than 1.4 Å to the native zinc water position. Sul500 O1 is at a distance of 3.3 Å from Thr199 N and Sul500 O3 2.6 Å from Thr199 Oγ1. The fourth sulfate O atom makes a van der Waals contact with Trp209 Cζ2 (3.1 Å).

4. Discussion

All the metal-substituted derivatives had unexpectedly bound ligands in their active sites, presumably as a result of the interference from *kupfernickels* (Nebergall, Holtzclaw & Robinson, 1980). The interpretations were complicated by the occupancy being only partial in the cases where sulfate binds (the cobalt enzyme at low pH, the nickel and manganese enzymes) and the lack of identification of the ligand molecules in the cobalt and copper enzyme. What can be said with certainty, however, is that the native tetrahedral zinc water position is occupied only in the cobalt enzyme and not by the other metal derivatives. At pH 7.8 this is because of a water molecule and at pH 6.0 by a sulfate O atom. In the copper, nickel and manganese derivatives, the coordination number and geometry is increased to either five or six. The active-site solvent structure is further

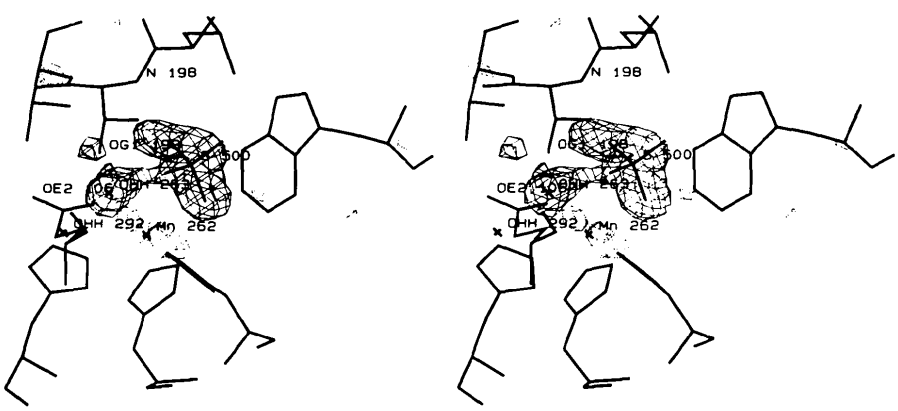

Fig. 7. The 'active site' of manganese(II)-substituted carbonic anhydrase at pH 7.8. Difference electron maps were calculated after refinement of native coordinates without waters 263, 338 and 318. Positive (continuous lines) and negative (broken lines) $|F_o| - |F_c|$ contours were drawn at $+3\sigma$. The occupancy of the sulfate was refined to 0.4.

disturbed in these three non-tetrahedral cases by the absence of water 318. In the native enzyme, both the zinc water and water 318 are hydrogen bonded to each other and to other molecules with tetrahedral geometry. The cobalt enzyme behaves in this respect as the native zinc enzyme in the two analyzed structures. Tetrahedral coordination for the cobalt-substituted bovine carbonic anhydrase II (CoBCAB) was also found both at high and low pH by spectroscopic methods, although cobalt human carbonic anhydrase I contained a large percentage of penta-coordinated species (Bertini & Luchinat, 1983).

It was suggested a few years ago that only hydrogen-bond donor atoms could bind at the tetrahedral zinc water position (Eriksson, Kylsten, Jones & Liljas, 1988), because of the hydrogen-bond pattern of Thr199 Oγ1. This is hydrogen bonded to Glu106 and the zinc water. Since the glutamate is believed to be deprotonated at physiological pH, it can only act as hydrogen-bond acceptor. Thr199 is therefore a hydrogen-bond donor to the glutamate and consequently an acceptor towards the zinc water. This theory was subsequently confirmed by the study of a number of carbonic anhydrase ligand complexes using X-ray crystallography (Liljas, Håkansson, Jonsson & Xue, 1993). Thus far only a couple of ligands with soft atoms have violated this rule and are bound with distorted geometry to the zinc ion and with increased distances between ligand and Thr199 Oγ1 (Jönsson, Håkansson & Liljas, 1993). Some implications of this hydrogen-bond chain on the catalytic function of the enzyme were suggested by Håkansson, Carlsson, Svensson & Liljas (1992), and Xue, Liljas, Jonsson & Lindskog (1993) demonstrated by site-directed mutagenesis that it was indispensable for the enzymatic activity.

In the cobalt enzyme at low pH and in the manganese enzyme, a sulfate O atom is within hydrogen-bonding distance of Thr199 Oγ1, but the protonization state of the liganded sulfate is unclear. The high concentration of sulfate (3 *M*) results in a concentration of approximately 0.3 m*M* of the monoprotonated species at pH 6 and 3 μM at pH 7.8, if we assume that the high ionic strength does not severely affect the dissociation constants of sulfate. Such a binding between monoprotonated sulfate and cobalt will require a rather strong binding constant, which is not unlikely in view of the higher affinity of the cobalt enzyme for some proton donor anions as compared to the native enzyme (Bertini, Luchinat & Scozzafava, 1982). Another, less likely, possibility is that the introduction of cobalt in combination with the high ionic strength of the ammonium sulfate solution alters the pK_a of Glu106 above its normal 'aqueous' value of 4.25 (Bertini, Lanini, Luchinat & Raciti, 1984). Thus, if we assume that the carboxylate group (*i.e.* the Oε1) of Glu106 is at least partly protonated at pH 6.0 under our experimental conditions, it can act as a hydrogen-bond donor to the Oγ1 of Thr199, enabling the latter to donate its H atom to the sulfate ion in the active site. The enzymatic activity of the cobalt enzyme is small at pH 6 but substantial at physiological pH (Kogut & Rowlett, 1987). In any case, whether a proton is bound to the sulfate or to Glu106, a net charge of -1 is added to the active site upon sulfate binding.

Sulfate binding has not been observed in any of the investigated native zinc enzyme structures (Liljas, Håkansson, Jonsson & Xue, 1993, and references therein). In contrast, spectroscopic and kinetic studies have suggested sulfate binding to both native and cobalt-substituted carbonic anhydrase (Lindskog, 1982; Simonsson & Lindskog, 1982; Pocker & Miao, 1987; Bertini, Canti, Luchinat & Scozzafava, 1977). Simonsson & Lindskog (1982) found that the esterase inhibition of sulfate never exceeded 20%, since the dissociation constant apparently increased with increasing sulfate concentration (ionic strength). Such a low degree of binding is difficult to observe crystallographically.

In conclusion, Nature's choice of zinc as the active site metal ion has probably been directed by its coordination chemistry, which favours tetrahedral coordination to a higher degree than any of the divalent transition metals. In the proposed mechanism (Håkansson, Carlsson, Svensson & Liljas, 1992) the coordination varies from 4 (tetrahedral) to 5 or 6 (distorted octahedral) depending on the definition of a ligand. The different states must be of roughly similar energy if trapped intermediates or unnecessary high peaks or deep troughs along the reaction coordinates are to be avoided. Cobalt also meets the requirements but the reaction is slowed down because the energy of state 5 in the proposed mechanism is too low relative to the other states (Yachandra, Powers & Spiro, 1983). Zinc is probably the best candidate both in its coordination chemistry, its natural abundance and its weaker interaction with anions and other possible ligands that might disturb the enzyme activity *in vivo*.

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