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# Laue and Monochromatic Crystallography on Carbonic Anhydrase

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# Laue and monochromatic crystallography on carbonic anhydrase

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[Plate 1]

We wanted to analyse the binding mode of small anion inhibitors to the zinc enzyme carbonic anhydrase in order to explore the binding of substrates and the catalytic mechanism of the enzyme. This was started by recording two data-sets by Laue diffraction to obtain the wanted structural information. In addition we wanted to test the capacity of the Laue method to show the small structural changes that are often associated with the catalytic activity of many enzymes. To be able to exploit fully time-resolved crystallography the method should be able to detect such minor structural changes. The obtained Laue results did not agree with the expected molecular structures. Thus we needed to record monochromatic data-sets of the same states of the enzyme to confirm our results. All major findings from the Laue data agree with the monochromatic data.

Stimulated by the unexpected findings we have continued the investigations of anion binding to carbonic anhydrase. We have studied both the zinc enzyme and replaced the native metal by cobalt which also yields an active enzyme. The accumulated picture of the ligand binding to the enzyme sheds new light on the substrate binding and on the catalytic mechanism.

## 1. Biochemical background

Carbonic anhydrase is an enzyme that early on was found to be inhibited by anions, and among them cyanide, which had been termed a metal poison (Meldrum & Roughton 1933). Subsequently the enzyme was found to contain stoichiometric amounts of zinc which made it natural to assume that cyanide binds to the metal (Keilin & Mann 1939, 1940). The zinc ion was found to be essential for the activity of the enzyme (Lindskog & Malmström 1962) and could be substituted by a number of other metals but only cobalt showed any significant activity (Rickli & Edsall 1962; Lindskog & Malmström 1962). The cobalt enzyme has since then been important for the studies of the binding of inhibitors and substrates due to its spectral properties (Lindskog 1963; Bertini & Luchinat 1982).

Isoenzyme II (CA II) is one of the most rapid enzymes found with a $k_{cat}$ around $10^6\ s^{-1}$ (Khalifah 1971). The reaction catalysed can be divided into three partial reactions (Silverman & Lindskog 1988):

$$E.OH^- + CO_2 + H_2O = E.H_2O + HCO_3^-, \quad (1)$$

$$E.H_2O = H^+.E.OH^-, \quad (2)$$

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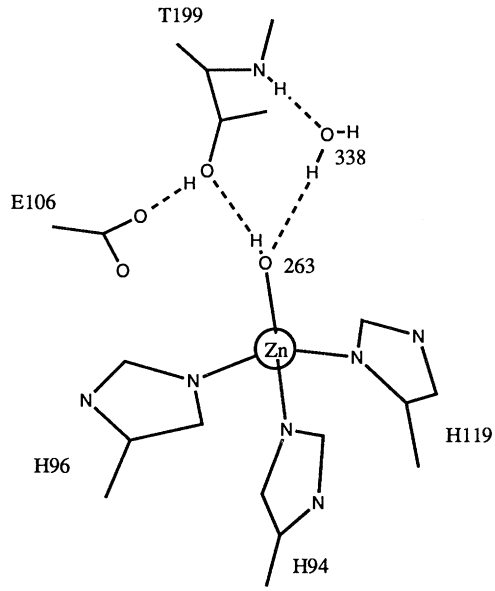


Figure 1. The structure of the active site of human carbonic anhydrase II. The zinc ion is tetrahedrally coordinated by three imidazole nitrogens and a water molecule (Wat 263). Thr199 is hydrogen bonded to Glu106. Ligands to the tetrahedral position of the zinc must be hydrogen bond donors to O$\gamma$1 of Thr199. In this way Thr199 functions as a doorkeeper preventing unprotonated anions to bind in the tetrahedral position of the metal. Wat338 is the 'deep' water which is replaced by most anion inhibitors.

$$H^+.E.OH^- + B = E.OH^- + BH^+. \quad (3)$$

Obviously the pH determines the direction of the reaction and the protonation state of the enzyme is important for the catalytic activity. The $pK_a$ value of the activity linked group is around 7 (Kernohan 1964; Lindskog 1982). The cobalt spectrum of the enzyme is clearly pH dependent with the same $pK_a$ as the one related to the activity (Lindskog 1963). Thus it has been a natural conclusion that a water molecule or hydroxyl ion bound to the metal is the one participating in the catalysis (Lindskog & Coleman 1973).

Carbonic anhydrase is inhibited by a number of simple anions as well as by aromatic sulphonamides and several other types of molecules. Most of these inhibitors are observed to affect the spectrum of cobalt carbonic anhydrase (Bertini *et al.* 1982). They concluded that some inhibitors yield a tetra-coordinated cobalt spectrum whereas others are best interpreted as penta-coordinated cobalt when compared to model compounds containing cobalt (Bertini & Luchinat 1982). Some compounds gave spectra of intermediate nature. EXAFS studies of the cobalt enzyme have confirmed those conclusions (Yachandra *et al.* 1983). The uninhibited cobalt enzyme has a tetrahedral spectrum at pH-values above 7 whereas an intermediate between four- and five-coordination is obtained below pH 7 (Bertini & Luchinat 1982).

The substrate binding to the enzyme is weak (Khalifah 1971). Nevertheless one has been able to observe that $HCO_3^-$ gives a cobalt spectrum which is interpreted as an intermediate between tetra- and penta-coordination (Bertini & Luchinat 1982). The variation in metal coordination and its relation to the catalytic mechanism has been

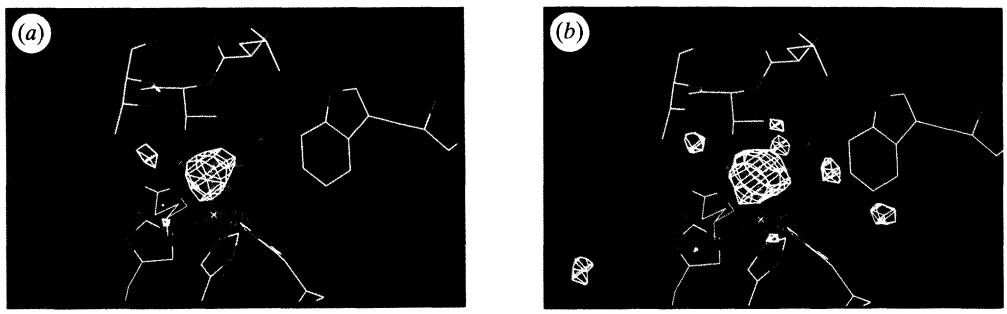


Figure 3. Human CA II at low pH. The zinc water was excluded from the refinement and the difference electron density maps unambigously shows the water in the tetrahedral position. (*a*) Laue data; (*b*) monochromatic data. The program O (Jones *et al*. 1991) was used on an Evans & Sutherland ESV graphical station.

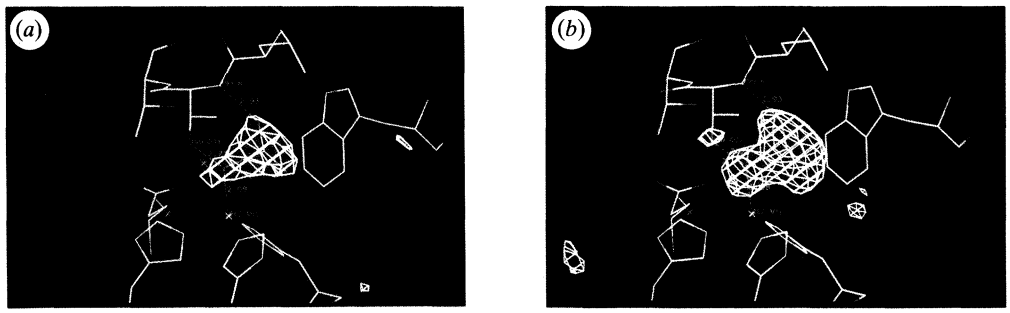


Figure 4. Human CA II in complex with bisulphite. After refinement of the native structure without the zinc water (Wat 263) and the deep water (Wat 338) the difference electon map shows the binding of the bisulphite. (*a*) Laue data; (*b*) monochromatic data.



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Table 1. *Comparison of data collection and refinement for Laue (Lindahl et al. 1992a) and monochromatic (Håkansson et al. 1992) data for human CA II at pH 6 and in complex with* $HSO_3^-$

| | pH 6 | | $HSO_3^-$ | |
|---|---|---|---|---|
| | Laue | mono | Laue | mono |
| ligand conc/mM | — | — | 300 | 300 |
| data collection | | | | |
| resolution/Å | 2.2 | 1.67 | 2.2 | 1.67 |
| time for data collection | 2 min | 3 days | 1 min | 3 days |
| completeness (%) | 55 | 87 | 67 | 89 |
| $R_m$ | 7.5 | 5.4 | 9.4 | 5.1 |
| refinement[a] | | | | |
| number of reflexions used | 7629 | 25425 | 9320 | 26190 |
| protein atoms | 2039 | 2060 | 2039 | 2069 |
| solvent atoms | 160 | 220 | 155 | 221 |
| $R_{cryst}$ (%) | 18.7 | 15.3 | 18.9 | 15.2 |
| positional error[b]/Å | 0.20 | 0.14 | 0.20 | 0.14 |
| RMS values | | | | |
| bond distance/Å | 0.019 | 0.021 | 0.022 | 0.021 |
| angle distance/Å | 0.034 | 0.037 | 0.037 | 0.037 |
| fixed dihedral distances/Å | 0.032 | 0.048 | 0.035 | 0.049 |
| out of plane restraints/Å$^2$ | 0.008 | 0.019 | 0.009 | 0.019 |
| chiral volume deviation/Å$^3$ | 0.118 | 0.233 | 0.132 | 0.234 |
| structure | | | | |
| coordination of metal | 4 | 4 | 4 | 4 |
| unrestrained distance from metal to solvent atom/Å | 1.07 | 2.05 | 2.0 | 2.09 |

[a] Restrained least squares refinement (Hendricksson & Konnert 1980) was carried out with the program PROFFT (Finzel 1987).
[b] From Luzzati plots (Luzzati 1952).

poorly understood. The different inhibitors will naturally reflect different ways of blocking the catalysis and crystallographic studies of their mode of binding should then give information about the function of the enzyme.

## 2. Previous crystallographic studies

The crystallographic analysis of the human isoenzyme II has led to a high-resolution structure of the enzyme (Liljas *et al.* 1972) which subsequently has been refined (Eriksson *et al.* 1988*a*; Håkansson *et al.* 1992). The zinc ion is bound by three imidazole nitrogens (His 94, His 96 and His 119) and one water molecule or hydroxyl ion (Wat 263). The metal coordination is close to tetrahedral (figure 1).

The studies of sulphonamide inhibitors (Liljas *et al.* 1972; Eriksson *et al.* 1988*b*; Vidgren *et al.* 1990) have shown a tetrahedral metal coordination as for the native enzyme. The first thorough study of a complex of an anion inhibitor was for $SCN^-$ (Eriksson *et al.* 1988*b*). The metal was observed to be penta-coordinated as expected from the cobalt spectrum (Bertini & Luchinat 1982). The electron density maps clearly show the zinc ion to be ligated by both the inhibitor and a water molecule (Eriksson *et al.* 1988*b*). The ligands give the metal a coordination geometry close to a trigonal bipyramide.




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The binding site of the thiocyanate gave new structural insight into the binding of inhibitors to the active site. A threonyl residue (199) is situated with its O$\gamma$1 hydroxyl near the zinc ion (3.9 Å†). This atom is hydrogen bonded to the carboxyl group of Glu106 which most certainly is charged around pH 8 where most of our investigations have been performed (Eriksson *et al.* 1988*b*; Merz 1991). Thus the threonyl residue must act as a hydrogen bond donor to the glutamate (figure 1). Residues bound at the tetrahedral position of the zinc ion are normally no further away from the threonyl oxygen than 2.7 Å and must therefore donate a hydrogen bond to it. In this way the threonyl residue functions as a 'doorkeeper', discriminating between protonated and non-protonated ligands. $SCN^-$ has no hydrogen and can therefore not bind at the tetrahedral position. It is instead situated at van der Waals' distance from O$\gamma$1 of Thr199, 2.2 Å from the zinc ion. This leaves enough room for a water molecule to bind at a new position at the zinc ion and donate a hydrogen bond to the threonyl residue.

We wanted to further explore the penta-coordinated state of the enzyme to shed further light on the mechanism of the enzyme. Guided by the cobalt spectroscopy we have chosen bisulphite and the enzyme at low pH as interesting examples. Devoid of data collection equipment for some time in the home laboratory and interested in the developing Laue method we decided to use this technique. The structural details especially in the case of the low pH study would at the same time be a test of the capacity of the Laue method to detect minor structural differences such as often will be found during enzyme catalysis. The results were unexpected in that both complexes showed a tetrahedral coordination of the zinc ion (Lindahl *et al.* 1992*a*). This was confirmed by subsequent monochromatic studies (Håkansson *et al.* 1992) and prompted further studies of inhibitor binding to the zinc (Lindahl *et al.* 1992*b*; Håkansson *et al.* 1992) as well as to the cobalt enzyme (Håkansson & Wehnert 1992). One basic conclusion is important for the understanding of the enzyme, but not surprising: zinc and cobalt are different!

## 3. The enzyme in complex with $HSO_3^-$ and at pH 6, a comparison of Laue and monochromatic data collection

### (*a*) *Data collection and refinement*

The Laue diffraction data were collected at SERC's Synchrotron Radiation Source (SRS), Daresbury. Stations 9.7 and 9.6 were used (Lindahl *et al.* 1992*a*). The data sets of pH 6 and $HSO_3^-$ were collected on films from one and two crystals respectively. The maximal resolution was 2.2 Å. The completeness of the data was good (66 and 81% respectively) for the interval 2.2–4.4 Å but due to harmonic overlaps the completeness was only 16% for both data-sets for the data at lower resolution than 4.4 Å. The overall completeness for each set was 55 and 67% respectively.

The monochromatic data-sets were collected using a Siemens area detector mounted on a Rigaku rotating anode (Håkansson *et al.* 1992). The maximum resolution was 1.67 Å. Two orientations of one crystal were used in both cases leading to a high completeness (87 and 89% respectively) of the data-sets. A comparison of the data collection and refinement using Laue and monochromatic data for the studies of the enzyme at low pH and the bisulphite complex is shown in table 1. Table 2 shows the agreement between the data-sets. Figure 2 shows a comparison of the

† 1 Å = $10^{-10}$ m = $10^{-1}$ nm

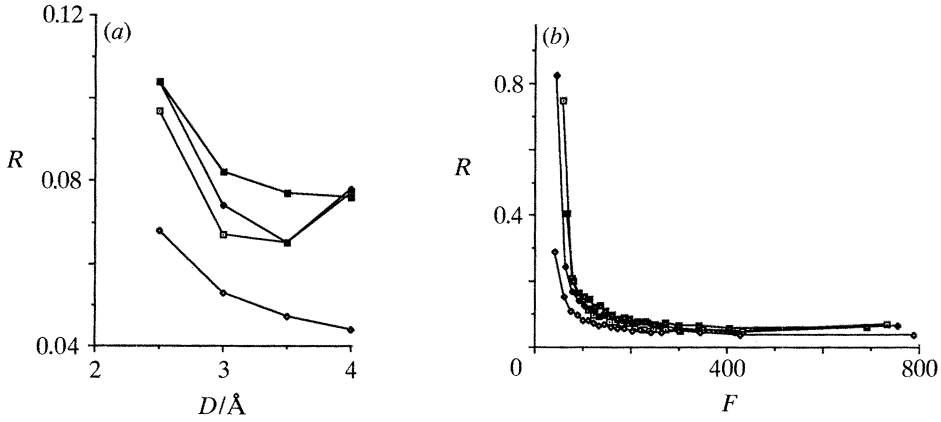


Figure 2. Comparisons of Laue and monochromatic data sets. (a) A plot of $R$ against resolution. (*b*) A plot of $R$ against F. The $R$ value plotted was $R = \Sigma||F_a|-|F_b||/\Sigma|F_a|$, where $|F_a|$ and $|F_b|$ are structure amplitudes in the various intervals. (⊡) describes the differences between the two data sets at pH 6; (◆) for the two data sets of the complex with $HSO_3^-$; (◘) for the two Laue data sets; (◇) for the two monochromatic data sets.

Table 2. *Agreement between Laue and monochromatic data-sets (resolution 2.2–4.4* Å)

| data-sets | $R$ (%) |
|---|---|
| $mono_{pH6}$–$mono_{HSO_3^-}$ | 5.4 |
| $Laue_{pH6}$–$mono_{pH6}$ | 8.0 |
| $Laue_{HSO_3}$–$mono_{HSO_3^-}$ | 8.4 |
| $Laue_{pH6}$–$Laue_{HSO_3^-}$ | 8.9 |

data-sets as a function of the resolution and the structure amplitude. Evidently for the strong reflexions there is good agreement but the weak reflexions are less accurately measured. In particular there is a large difference for these reflexions in comparing Laue with monochromatic data.

The starting coordinates in the refinement were in all cases those of Eriksson *et al.* (1988*a*). In all the complexes studied a minimum of four water molecules were initially removed from the immediate environment of the zinc ion before least squares refinement of the atomic coordinates was performed. Subsequent electron density maps gave clear indications of what structures should be inserted.

The differences in the results from the refinement of the Laue and monochromatic data (table 1) mainly reflect the differences in resolution and completeness of the data as well as differences in the restraints in the refinement. With the monochromatic data about sixty additional solvent molecules could be defined. In general the new water molecules have higher temperature factors (on average 45 Å$^2$ in comparison to 30 Å$^2$ for the initially characterized water molecules) and less well defined electron density. A few residues with high temperature factors at both termini as well as some residues with multiple conformations were identified in the monochromatic maps. One residue in the original set of coordinates, Asn 253, was not fitted in the best way to the electron density. This was evident from a significant negative difference electron density also in the Laue maps. It was, however, not possible to identify the correct conformation of the residue from the Laue maps.

(*b*) *Structural results*

$|F_o|-|F_c|$ maps from the Laue data of the enzyme at pH 6 (figure 3*a*, plate 1) showed one single density at the zinc ion (Lindahl *et al.* 1992*a*). The position was in full agreement with the zinc water position at pH 8.5 (Eriksson *et al.* 1988*a*; Håkansson *et al.* 1992). The monochromatic data measured later (figure 3*b*, plate 1) confirmed this observation (Håkansson *et al.* 1992). Nair & Christianson (1991) have obtained the same result.

The $|F_o|-|F_c|$ maps calculated from the Laue data of the bisulphite complex (figure 4*a*, plate 1) showed a triangular density at the zinc ion (Lindahl *et al.* 1992*a*). This was best interpreted as a bisulphite ion with one of its oxygens at the position of the zinc water and one of the others at a distance of 3.3 Å away from the zinc ion. The monochromatic data (figure 4*b*, plate 1) again confirmed these results (Håkansson *et al.* 1992).

Without the possibility to observe the hydrogen atoms these results are consistent with our hypothesis in which only protonated atoms can bind at the tetrahedral position of the zinc. In the case of the enzyme at low pH a water molecule has replaced the hydroxyl ion bound at higher pH values. No tendency of penta-coordination is noticed. In the case of the bisulphite binding the proton of the inhibitor is presumably situated on the zinc bound oxygen which is involved in the hydrogen bond to O$\gamma$1 of Thr 199.

## 4. Further studies of anion binding

The main data about the binding of anions to the enzyme has until recently originated from spectroscopy on the cobalt substituted enzyme. We decided that a continued analysis of the crystallography of the zinc enzyme was needed to clarify the mode of binding for anions. Two anions were particularly difficult to understand. Cyanide and cyanate have been found to bind to the metal with tetrahedral coordination from various spectroscopic studies (Bertini & Luchinat 1982; Yachandra *et al.* 1983). They are also supposed to bind as the negatively charged species (Feeney *et al.* 1973; Kanamori & Roberts 1983). This is incompatible with our mentioned hypothesis of the doorkeeper function of Thr 199 and was therefore subject to investigation. Monochromatic data has been recorded for cyanide and cyanate to 1.8 and 1.6 Å respectively and refined to crystallographic $R$ values of 16% (Lindahl *et al.* 1992*b*). In neither case are the ions observed to bind at the metal but near it and the zinc water. They are replacing a water molecule in the deep end of the active site, the 'deep' water (Wat 338) and are hydrogen bonded to the amide of Thr 199. The position of the zinc water is maintained and the inhibitors come no closer to the zinc ion than 3.2 Å. The coordination is thus tetrahedral. Again the hypothesis about the doorkeeper function of Thr 199 could be strengthened.

Without going into further details a brief summary of some of the complexes that have been studied is given in table 3. As can be seen the complexes studied so far are consistent with the doorkeeper hypothesis. Some of the inhibitors are no closer to the metal than 2.5 Å (formate; Håkansson *et al.* 1992) or 2.8 Å (nitrate; Mangani & Håkansson 1992). The reason for classifying them as four- to five-coordinated is that the geometry of the zinc has been altered from a tetrahedron towards a trigonal bipyramide. Another feature of interest is that all changes in protein structure at binding of inhibitors are very small, on the limit of being significant.





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Table 3. *Anion complexes of human CA II studied*

| complex | resolution Å | metal coordination spectr.(a) | metal coordination X-ray | proton donor to Oγ1 of Thr199 | metal ligand* | ref. |
|---|---|---|---|---|---|---|
| pH 7.8 | 1.5 | 4 | 4 | $OH^-$ | $OH^-$ | b |
| pH 6 | 1.7 | 4–5 | 4 | $H_2O$ | $H_2O$ | b, c |
| $RSO_2NH^-$ | 1.9 | 4 | 4 | $NH^-$* | $NH^-$* | d |
| $SCN^-$ | 1.9 | 5 | 5 | $H_2O$ | $H_2O$+N* | e |
| $HSO_3^-$ | 1.7 | 5 | 4 | OH* | OH* | b, c |
| $CN^-$ | 1.8 | 4 | 4 | $H_2O$ | $H_2O$ | f |
| $OCN^-$ | 1.6 | 4 | 4 | $H_2O$ | $H_2O$ | f |
| $HCOO^-$ | 1.9 | 5 | 4–5 | $H_2O$ | $H_2O$+O* | b |
| $NO_3^-$ | 1.9 | 5(g) | 4–5 | $H_2O$ | $H_2O$+O* | h |
| $SH^-$ | 1.9 | 4 | 4 | $SH^-$* | $SH^-$* | h |
| T200H + $HCO_3^-$ | 1.9 | 4–5 | 4–5 | OH* | OH*+O* | i |

* atom(s) of the inhibitor.

a, Bertini & Luchinat 1982; b, Håkansson *et al.* 1992; c, Lindahl *et al.* 1992*a*; d, Vidgren *et al.* 1990; e, Eriksson *et al.* 1988*b*; f, Lindahl *et al.* 1992*b*; g, Bertini *et al.* 1982; h, Mangani & Håkansson 1992; i, Xue *et al.* 1992*b*.

In addition to the anion complexes mentioned a few mutants have been investigated that further support the model described above. A mutation of Glu 106 to Gln destroys the doorkeeper function by altering the obligatory hydrogen bond arrangement. Crystallographic analysis (Xue *et al.* 1992*a*) shows that the zinc ion under these circumstances binds a sulphate ion from the crystallization medium. In the wild-type protein the doorkeeper prevents this inhibitory action.

## 5. Cobalt carbonic anhydrase

All the results described above are compatible with the role of Thr 199 as a doorkeeper that prevents non-protonated ligands from binding at the tetrahedral position of the zinc. However, there is a discrepancy between the spectroscopy on the cobalt enzyme and the crystallography on the zinc enzyme in the case of pH 6 and bisulphite. There is also a discrepancy with regard to cyanide and cyanate that were supposed to bind at the metal. We wanted to investigate whether these differences originated from the difference between zinc and cobalt.

We have performed high-resolution studies of a number of states and complexes of the cobalt enzyme. We have so far met with a number of difficulties. The first problem with cobalt is the uncertainty of the oxidation state. This ambiguity was resolved by dissolving crystals of the cobalt enzyme. Analysis of the spectrum and of the enzymatic activity showed that the metal of the crystals is $Co^{2+}$ and not $Co^{3+}$ (Håkansson & Wehnert 1992). Secondly, all the crystallographic studies have been performed in ammonium sulphate which has a concentration of at least 2.4 M. This means that ammonia and sulphate ions will compete for the binding at the metal. This did not appear as a problem in the studies of the zinc enzyme. At high pH there is no significant difference between the zinc and cobalt enzymes. However, at pH 6 a large density that we have interpreted as a fractional occupancy of an $HSO_4^-$ ion is bound at the cobalt. In the case of the zinc enzyme the same amount of sulphate was present without detectable binding to the metal. Two conclusions can be reached. The first is that cobalt and zinc behave differently with cobalt having higher affinity for various ligands. The second conclusion is that to be able to examine the

differences between zinc and cobalt we need to do the studies in a medium different from ammonium sulphate.

## 6. Conclusions

The knowledge about the mode of binding for a number of anion inhibitors of carbonic anhydrase has been extended. We have also clearly established that Thr 199 and Glu 106 are essential for the location and orientation of metal ligands. This has implication for the binding of bicarbonate in the active site. Instead of binding to the metal with a negatively charged oxygen the doorkeeper requires that the protonated oxygen is bound (Xue *et al.* 1992*b*). This has obvious consequences for the catalytic mechanism as discussed by Håkansson *et al.* (1992).

The Laue method that initially led us onto the present studies can obviously distinguish between subtle differences such as occur in catalytic mechanisms and for which time resolved Laue crystallography is needed. All efforts to increase resolution and completeness of data are of course as in all types of crystallography very important.

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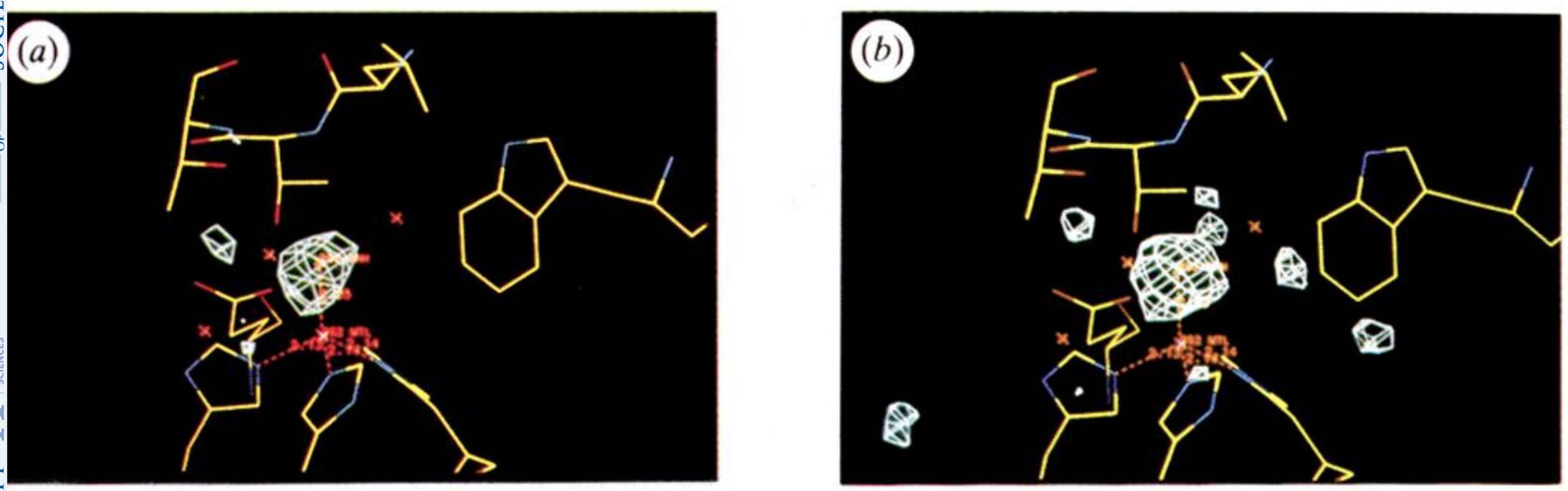
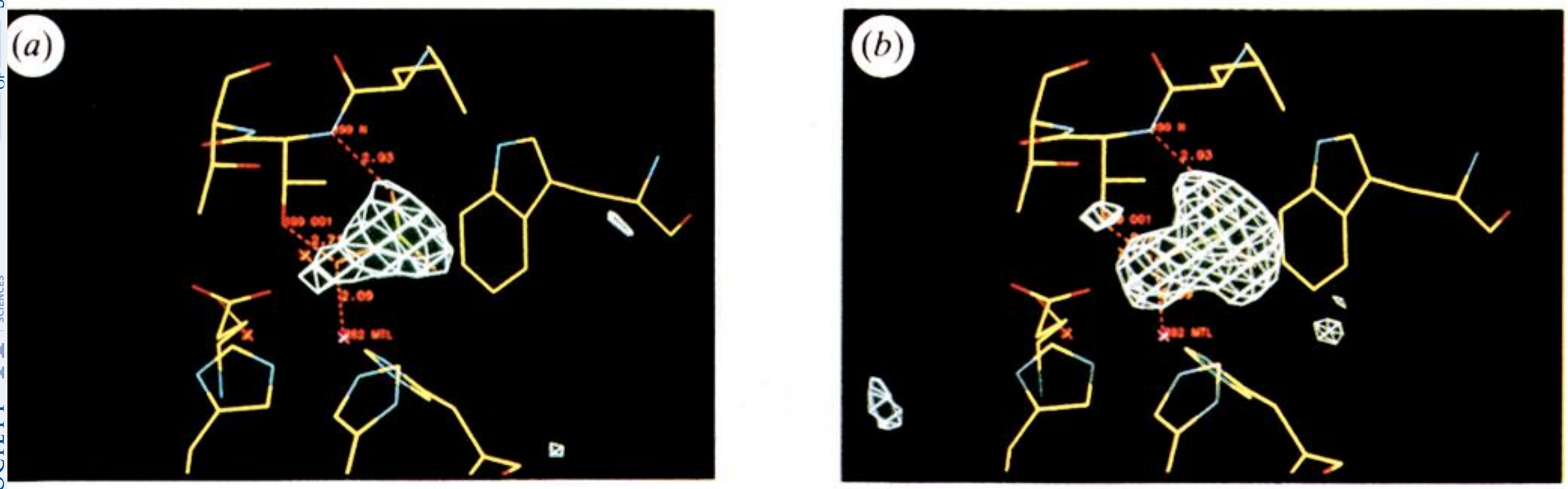



igure 3. Human CA II at low pH. The zinc water was excluded from the refinement and the differ-
nce electron density maps unambigously shows the water in the tetrahedral position. (*a*) Laue data;
) monochromatic data. The program O (Jones *et al*. 1991) was used on an Evans & Sutherland ESV
raphical station.




igure 4. Human CA II in complex with bisulphite. After refinement of the native structure without the zinc water (Wat 263) and the deep water (Wat 338) the difference electon map shows the inding of the bisulphite. (*a*) Laue data; (*b*) monochromatic data.