STRUCTURE AND FUNCTION OF CARBONIC ANHYDRASES

Imidazole binding to human carbonic anhydrase B and the mechanism of action of carbonic anhydrases

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Received 25 November 1976

1. Introduction

The isoenzymes carbonic anhydrases B and C (EC 4.2.1.1. $CO_2+H_2O \rightleftarrows HCO_3^- + H^+$) from human erythrocytes have been extensively investigated by different physico–chemical methods in order to understand the mechanism of action of these efficient enzymes [1–4]. These two enzymes differ in their catalytic rate by a factor of about 5 for a number of reactions investigated. The primary structures of these isoenzymes exhibit a high degree of homology and so do their tertiary structures [3].

Khalifah [5] has shown that imidazole is a competitive inhibitor of the hydration reaction of CO_2 by human carbonic anhydrase B. This is the only competitive inhibitor of this reaction reported for any carbonic anhydrase. It has been reported by a number of workers that aromatic or heterocyclic sulphonamides are competitive inhibitors for the reverse dehydration reaction [1,2]. The availability of a competetive inhibitor is of great help in the investigation of the enzyme mechanism by X-ray diffraction methods. We have located the binding site of imidazole in human carbonic anhydrase B (HCAB) crystals and arrived at the most probable CO_2 binding site as well as the mechanism of action of the enzyme consistent with the existing data.

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2. Materials and methods

Crystals of the native B enzyme HCAB were prepared by the seeding technique described by Kannan et al.[6]. The native enzyme crystals were then dialysed in 2.4 M $(NH_4)_2SO_4$, 0.05 M Tris–HCL, 0.5 M imidazole at pH 8.7 for 2 weeks and used in the X-ray diffraction analysis. About 75% of the 2 Å data were collected by precession photography using Ni filtered Cu Kα-radiation. The photographs were measured on a fully automated microdensitometer and processed on an IBM 370/155 computer [7,8]. The phase angles of the native HCAB calculated by the isomorphous replacement method [9] were used to calculate the electron density map of the HCAB–imidazole complex and a difference Fourier map between the complex and the native enzyme using the Fourier program GNRFOUR, kindly provided to us by Dr G. N. Reeke Jr.

3. Results

The difference Fourier (fig.1,2) contained only two positive peaks, one major and one minor, localized in the active site region. The electron density map of the HCAB–imidazole complex (fig.3) showed only one significant positive peak which coincided with the major peak in the difference map. This major peak is located near the zinc ion (fig.2,3). Using Kendrew-Watson skeletal model parts and an optical comparator [10] we have built the imidazole

Fig.1. Composite pictures of the electron density maps of native human carbonic anhydrase B and the difference Fourier map of the imidazole–HCAB complex. Only a part of the active site is shown. Arrows indicate the peaks (lighter thick contours) in the difference Fourier. The major peak is located near the essential zinc ion distinguishable by the high electron density. The minor peak is located on an electron density peak in the native enzyme that does not belong to the protein.

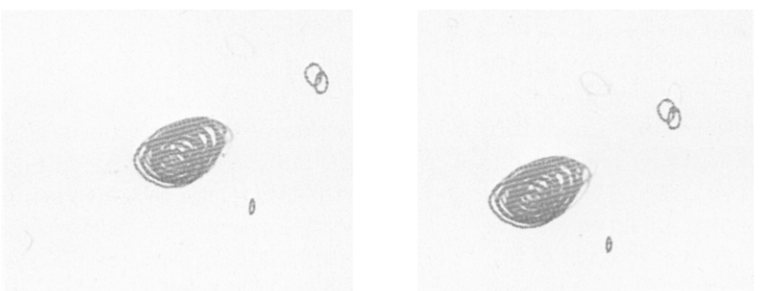

Fig.2. Difference Fourier map of HCAB–imidazole complex versus HCAB native enzyme. Sections around the active site stacked together.

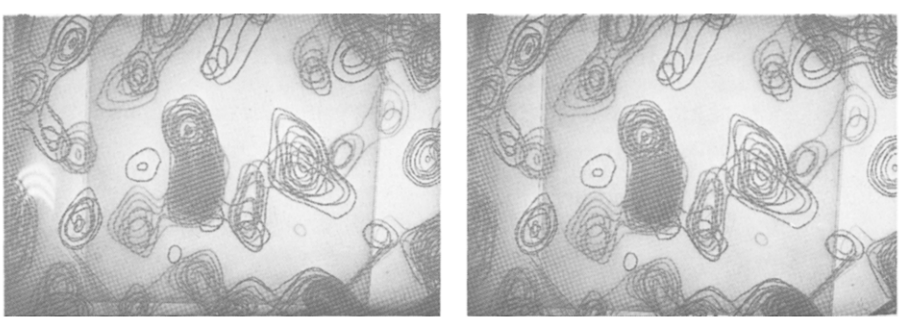

Fig.3. Electron density map of HCAB–imidazole complex. Sections around the active site stacked together and oriented as in fig.4.

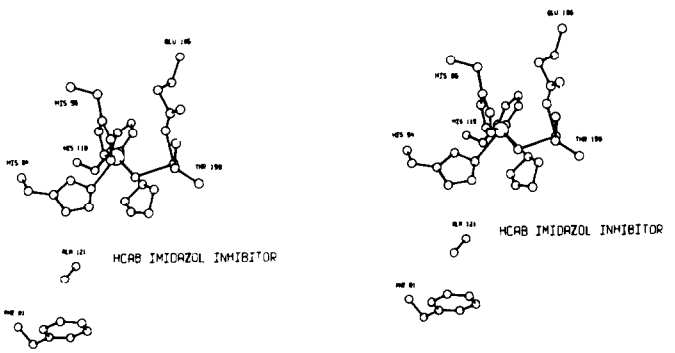

Fig.4. ORTEP drawing of the interpretation of the imidazole binding based on the difference Fourier and the electron density maps of HCAB–imidazole complex. See Results (3) and Discussion (4) for amino acid residue details.

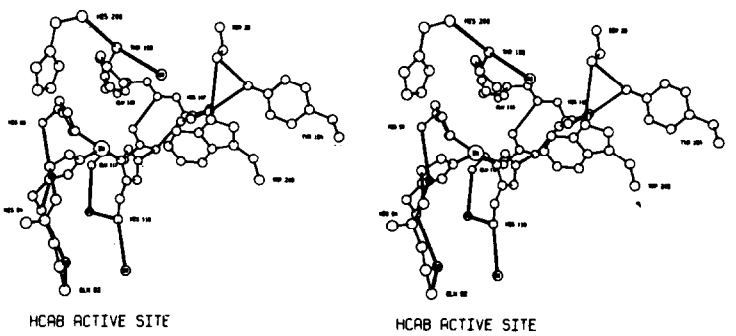

Fig.5. ORTEP drawing of the active site region of HCAB. Some of the residues in the active site are shown. See Results (3.1) for amino acid residue details.

ring in the electron density maps. The interpretation is shown in fig.4.

3.1. *Description of the active site of HCAB*

A brief description of the active site of HCAB is given here for the sake of completeness. For greater detail the reader is referred to Kannan et al. [8] and Notstrand et al. [3].

Some of the amino acid residues located in the active site of HCAB are shown in fig.5 together with the essential zinc ion. Notstrand et al. [3] have discussed the similarities and differences in the tertiary structure of the human carbonic anhydrase isoenzymes. In the native enzymes the metal ion is liganded to three histidyl residues, His 94, His 96 and His 119. A solvent molecule, water or hydroxyde ion, occupies the fourth liganded site giving the metal ion a distorted tetrahedral coordination. The metal coordinated solvent molecule is hydrogen bonded to Thr 199 which is hydrogen bonded to Glu 106 which is partially buried inside the active site cavity. One of the zinc ligands, His 119, is hydrogen bonded to Glu 118, which is in turn hydrogen bonded to His 105, both residues being buried in the interior of the protein (fig.5). Glu 118 is also hydrogen bonded to the amide nitrogen of His 105. Glu 118 would be charged at pH 8.7 where the crystal structure has been investigated and its charge may be compensated to a great extent by His 105 leaving the liganded histidyl residues uncharged. Thus the two positive charges of the zinc ion in carbonic anhydrase would not be fully compensated by its ligands which may be of importance for the function of the enzyme.

4. Discussion

Khalifah [5] has shown that modification of the nitrogen atoms of the imidazole destroys its inhibitory property towards carbonic anhydrase B. Imidazole binding also produces characteristic changes in the visible absorption spectrum of cobalt CAB (unpublished results of P. A. Whitney as reported in [5]). Grell and Bray [11] have studied the EPR spectra of cobalt substituted bovine carbonic anhydrase in imidazole buffer. They found that the imidazole binds weakly to the enzyme through direct coordination to the metal ion. These results are validated by the X-ray diffraction analysis reported here. The nitrogen atom of the imidazole is about 2.7 Å from the zinc ion and is located in a possible fifth coordination site in a hydrophobic pocket. The presence of the electron density assigned to the fourth ligand to the metal ion and interpreted as a solvent molecule [9] in the electron density maps of the HCAB–imidazole complex and the absence of a negative peak in the difference Fourier at this site indicates that the imidazole binds to the metal without displacing the solvent molecule located as the fourth ligand. The plane of the imidazole ring is almost perpendicular to the plane of the metal liganded His 94 (fig.4). The latter observation is consistent with the interpretation proposed by Campbell et al. [12] on the basis of their NMR studies on HCAB–imidazole complex.

Sulfonamide and anionic inhibitors bind to the zinc replacing the liganded solvent molecule in both isoenzymes [13–15]. The hydroxyl of Thr 199 is hydrogen bonded to the oxygen or nitrogen of the sulfonamide group. Another oxygen atom of the sulfonamide group is located in the fifth coordination site of the metal ion [15], which is the same as that occupied by the nitrogen atom of the imidazole.

It is thus apparent from the structure of the inhibitor complexes of the carbonic anhydrases that the essential zinc ion has 4 nearly tetrahedral ligand directions and a somewhat distant fifth coordination site, a situation similar to that reported for some model compounds [16].

4.1. *CO_2 binding site and the mechanism*

Riepe and Wang [17] have proposed on the basis of difference infrared measurements that CO_2 is bound without appreciable strain to the enzyme in a hydrophobic pocket near the metal ion. They also showed that anionic inhibitors interfered with the CO_2 binding.

By analogy to the imidazole binding, we propose that CO_2 binds weakly to the fifth coordination site of the metal in the hydrophobic region of the active site. In this arrangement not only will the two substrates in the forward reaction be in a favourable orientation but the carbon atom to be hydrated will be at the right proximity to the hydroxyl liganded to the metal ion. The positively charged metal ion would polarize the CO_2 and increase the positive charge on the carbon favouring the formation of bicarbonate. The reactivity of the zinc bound solvent molecule might be altered favourably by its connection to Glu 106 via Thr 199 by hydrogen bonding, both these residues are known to be invariant in primate and ruminant carbonic anhydrases [18]. The activity-linked ionizing group with a pK_a near 7 could either be the zinc-bound water, as proposed by a number of investigators [1] and illustrated in fig.6, or Glu 106 as shown in fig.7. The latter mechanism requires an anomalous pK_a for Glu 106, but this residue is partially buried in the interior of the active site and would be expected to be shielded from the solvent.

The zinc hydroxide mechanism shown in fig.6 would proceed without any internal proton transfers

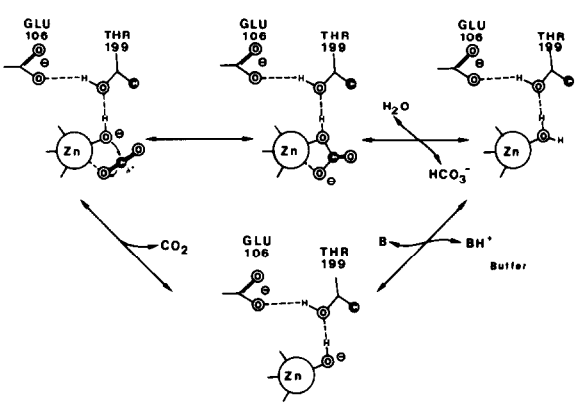

Fig.6. Proposal for the hydration–dehydration mechanism of carbonic anhydrase. The pK of the zinc bound water is envisaged to be lowered to about 7.0 by the charge distribution on the metal ion and also helped by Glu 106 by the hydrogen bonding through Thr 199.

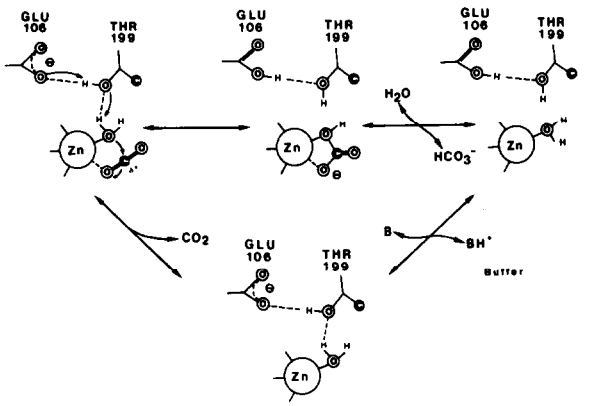

Fig.7. Alternative proposal for the mechanism where Glu 106 is envisaged to accomplish the proton transfer.

in contrast to the Glu 106 mechanism (fig.7), where the catalytic step involving the transformation of CO_2 into HCO_3^- is associated with internal proton transfers. Steiner et al. [19,20] observed substantial hydrogen isotope effects on the kinetic parameters of human carbonic anhydrase C and interpreted these effects to be associated with an internal proton transfer process in the free enzyme rather than in the $CO_2-HCO_3^-$ transformation step. Thus it would seem that the presently available data do not suffice to distinguish between these two alternatives. The ultimate proton transfer into the solvent medium is believed to be buffer mediated [21] as shown in figs.6 and 7.

Acknowledgements

We would like to thank Dr S. Lindskog for very instructive and fruitful discussions and valuable suggestions . We would like to acknowledge Professor B. Strandberg, Drs A. Liljas and I. Waara for useful discussions. We are very much indebted to Gunnel Johansson for typing the manuscript and Agneta Borell for preparing the stereo drawings.

This work was supported by grants from the Faculty of Science, University of Uppsala and the Swedish National Science Research Council.

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