Inorganica Chimica Acta, 46 (1980) L115–L117

A Two Site Mechanism for Carbonic Anhydrase B

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Received June 4, 1980

The isoenzymes carbonic anhydrase B and C from human erthrocytes are zinc metalloenzymes which have been the subject of much interest for many years. Extensive studies have shown that the reactions (1)–(3) take place at the same site

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

$$RCOOR' + H_2O \rightleftharpoons RCO_2^- + R'OH + H^+ \quad (2)$$

$$RCHO + H_2O \rightleftharpoons RCH(OH)_2 \quad (3)$$

Khalifah [1] has established that imidazole is a competitive inhibitor of the hydration of CO_2 by human carbonic anhydrase B. This is the only competitive inhibitor reported for any carbonic anhydrase. Aromatic or heterocyclic sulphonamides such as acetazolamide are competitive inhibitors for the reverse dehydration reaction [2, 3].

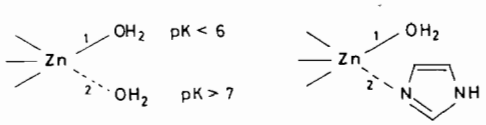
The native enzyme contains one mol of zinc(II), which can be removed by dialysis against pyridine-2,6-dicarboxylic acid [4]. The resulting apoenzyme can be used to prepare synthetic cobalt(II) [5], cobalt(III) [6]; copper(II) [3] and nickel(II) [3] enzymes. The copper and nickel carbonic anhydrases display only little catalytic activity, however, the cobalt(II) enzyme is active in CO_2 hydration [7] and has been used as a model for the native enzyme. The d^7 cobalt(II) enzyme is high spin and d–d spectrum is markedly pH-dependent [8–10]. The catalytic activity of both the native and cobalt(II) enzymes is also pH-dependent [7].

The pH-dependence of both the d–d spectrum and the catalytic activity of the cobalt(11) enzyme have been attributed to a single ionisation equilibrium [11–14], however, Bertini *et al.* [15] have recently shown that two ionisations with pK_a values of <6 and >7 are required to account for the pH dependence of the d–d spectrum. A recent ^{13}C NMR study on a chemically modified carbonic anhydrase has also indicated more than one ionisation in the active site cavity [16]; as has a study of hydrogen isotope effects on the activity of the human C enzyme [17]. The origins of these ionisations is controversial, having been attributed to co-ordinated water on zinc [8, 9], co-ordinated imidazole [18, 19] or possible E1u 106 [20].

The X-ray data at 2 Å resolution [21] has shown that the zinc ion is ligated by three histidyl residues, His 94, His 96 and His 119. A water molecule or hydroxide ion occupies a fourth site giving a distorted tetrahedral stereochemistry on zinc. The metal-co-ordinated solvent molecule is hydrogen bonded to Thr 199 which in turn is hydrogen bonded to Glu 106, partially buried inside the active site cavity.

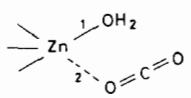
The purpose of the present communication is to consider a possible mechanism which will account for the available experimental observations on the enzyme and its metal substituted derivatives. Much of the data can be rationalised if it is assumed that there are *two sites* on zinc(II) or cobalt(II) carbonic anhydrase which can be occupied in addition to the three ligating histidine residues. Five-co-ordinate zinc(II) complexes are well documented, and such complexes have been used as biomimetic models for carbonic anhydrase [22]. For example, $Zn(terpy)Cl_2$ has a distorted trigonal bipyramidal stereochemistry, with the three nitrogen donors in one plane [23], the zinc–nitrogen bonds are 2.2 Å and the zinc–chlorine bonds 2.29 Å. $Zn(salen)H_2O$, has a distorted square pyramidal stereochemistry, the zinc(II) lying 0.34 Å above the salen plane, with the water molecule 2.13 Å below the zinc ion [24].

Kannan and co-workers [25] have recently described the X-ray structure of the imidazole–carbonic anhydrase B inhibitor complex. They have found that imidazole binds weakly to the enzyme by direct co-ordination to the metal ion. The pyridine nitrogen of imidazole is *ca.* 2.7 Å from the zinc and does not displace the solvent water molecule. In addition, imidazole binding produces characteristic changes in the d–d spectrum of cobalt(II)–carbonic anhydrase B [26], and epr studies of the cobalt(II) enzyme in imidazole buffers are consistent with the imidazole binding weakly to the metal ion [27]. The experimental data suggests that the co-ordination of zinc(II) can be represented as in (I) and the imidazole–carbonic anhydrase B inhibitor complex as in (II).



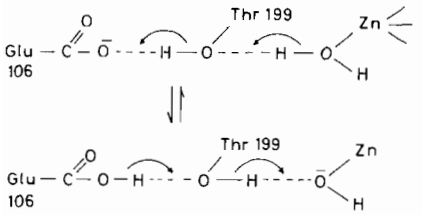
(I)

(II)



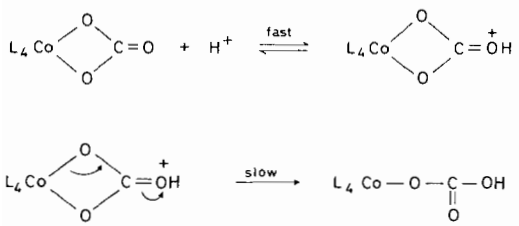
(III)

For the imidazole complex $[Co(Im)_6](NO_3)_2$, the M–N distance is 2.16 Å [28], while for $[Zn(Im)_6]Cl_2 \cdot 4H_2O$ the M–N distance is 2.15–2.26 Å [28]. Similar values have been obtained for other imidazole complexes of these metal ions [28]. The bond length of 2.72 Å in the imidazole–carbonic anhydrase B inhibitor complex indicates a relatively weak bond. As imidazole inhibits the hydration of CO_2 it is probable that the binding of CO_2 occurs at site 2 as in (III), in which there is a weak interaction between the substrate and zinc(II) leading to some Lewis acid catalysis. The Glu 106–Thr 199–(Zn-(OH_2)) sequence provides a charge relay system of the type suggested for chymotrypsin, to account for the enhanced nucleophilicity of Ser 195 at the active site of the enzyme [29]. The effect in carbonic anhydrase is to increase the nucleophilicity of the coordinated water or hydroxide ion (Scheme 1)

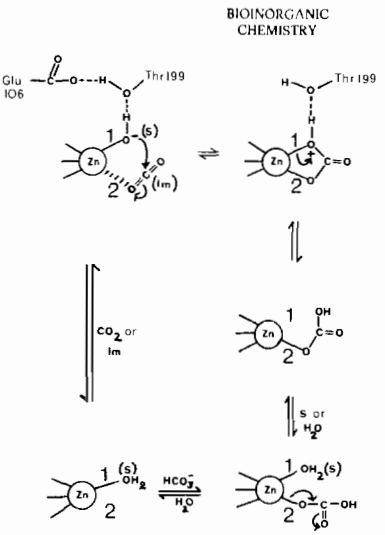


Scheme 1. Charge relay system

Co-ordinated hydroxide ion is recognised to be a very effective nucleophile in intramolecular reactions [30]. Attack of co-ordinated hydroxide (site 1) on co-ordinated CO_2 (site 2) gives chelated bicarbonate (Scheme 2). The opening of metal chelate rings is generally an unfavourable process and the opening of carbonato rings normally requires specific acid or specific base catalysis [31–33]. Recent investigations have shown that the mechanism of the acid-catalysed ring opening of cobalt(III) carbonato complexes of the type $[CoL_4CO_3]^{n+}$ involves pre-equilibrium protonation of the complex, followed by slow ring opening which may, or may not, involve water in the rate-determining step [31, 32]



The reaction is subject to a deuterium solvent isotope effect k_{D_2O}/k_{H_2O} of *ca.* 2.6 [31, 32]. In the enzyme system, Thr 199 can effectively provide intramolecular general acid catalysis for ring opening of the carbonato complex. Sulphonamides are competitive with water (or hydroxide iron) for site 1. Although bicarbonate can bind in site 2 its dehydration is inhibited by sulphonamide. Imidazole and CO_2 compete for site 2 and thus the hydration step is inhibited in the presence of imidazole. The availability of two catalytic sites on zinc can account for many of the experimental observations noted with the enzyme.



Scheme 2. Possible mechanism for carbonic anhydrase (S = sulphonamide, Im = imidazole).

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