The Kinetics and Mechanism of Zn(II) Dissociation from Human Carbonic Anhydrase B in the Presence of Chelating Agents

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Mammalian carbonic anhydrase is a metalloenzyme consisting of a single polypeptide chain containing one zinc cation [1]. X-ray crystallographic data for the human carbon anhydrase C isoenenzyme suggested that the zinc is 4-coordinate and bound to three histidyl residues and one water molecule [2, 3], however more recent kinetic evidence suggests that a fifth coordination site [4] might also be present.

The zinc cation is essential for enzyme activity and removal of it to produce the apoenzyme leads to deactivation; activity of the apoenzyme can be restored by addition of either zinc or cobalt ion [5]. The kinetics of dissociation of zinc or cobalt from the corresponding forms of bovine carbonic anhydrase (BCA) in the presence of various chelating agents has been studied by several groups [6–10]. Evidence suggests that in most cases the loss of metal takes place via a ternary complex involving the apoenzyme, metal ion and the chelating agent. However for zinc BCA in the presence of EDTA, it appears that the rate of removal of the zinc is governed by the rate of spontaneous dissociation of the zinc enzyme [8].

This paper presents the results of an investigation of the kinetics of removal of zinc from human carbonic anhydrase B by various chelating agents. The study makes possible a comparison of the dissociation of the human and bovine forms of the enzyme.

Experimental

2,6-Pyridinedicarboxylic acid (2,6-PDA), *p*-aminomethylbenzenesulphonamide-HCl and *p*-nitrophenyl acetate were obtained from Sigma Chemical Company, EDTA was obtained from Ajax Chemical Company. 1,10-Phenanthroline (phen) and 2,2′-bipyridine (bipy) were obtained from BHD, both were recrystallised before use.

Human carbonic anhydrase B (HCAB) was prepared from outdated human blood using a modification of the Osborne and Tashian method [11]. The affinity gel was prepared by coupling *p*-aminomethylbenzenesulphonamide-HCl to Affi-Gel 10 (Bio-Rad) Unreacted sites were blocked by ethanolamine and the gel equilibrated with 0.2 *M* Tris-SO_4 buffer pH 9. HCAB was eluted with 0.1 *M* Tris-SO_4 buffer pH 7.0 containing 0.4 *M* KI. The enzyme product showed a single band on SDS-gel electrophoresis with an apparent molecular weight of 29,000 [12]. The concentration of the enzyme was measured by the absorbance at 280 nm assuming a molar extinction coefficient of 4.73×10^3 M^{-1} cm^{-1}.

Carbonic anhydrase activity was determined at pH 7.5 using *p*-nitrophenylacetate as substrate [13]. A molar extinction coefficient of 1.39×10^4 M^{-1} cm^{-1} at 400 nm was used for *p*-nitrophenol.

The kinetics of removal of zinc from HCAB were studied at 4 °C. The reaction mixture (1 ml) consisted of HCAB (4.9×10^{-5} *M*) in 0.2 *M* sodium acetate buffer pH 5.0 and the required concentration (2×10^{-3}–5×10^{-2} *M*) of the appropriate chelating agent. Although the presence of acetate ion may influence the demetallation of carbonic anhydrase [7], acetate buffer was employed for the previous study involving BCA [8] and was retained in the present one in order that the results from both studies might be directly comparable. The ionic strength of the mixture was kept constant at 0.35 *M* by addition of NaCl. Aliquots (50 μl) were removed at appropriate times and assayed for carbonic anhydrase activity as described above. Controls containing no chelating agent were run alongside the test samples.

Results and Discussion

The inactivation of HCAB by removal of zinc from the holoenzyme was studied in the presence of large excess of the respective chelating agents to give pseudo-first order conditions and to force the following equilibrium in the direction of the apoenzyme

$$HCABZn + nL \rightleftharpoons \text{apo-HCAB} + ZnL_n$$

As mentioned previously, the removal of zinc from bovine carbonic anhydrase with the majority of chelating agents occurs through a ternary complex [6–10] with the cleavage of the bond between the metal ion and the protein being the rate determining step.

$$(CA)Zn + L \underset{}{\overset{K_{EML}}{\rightleftharpoons}} (CA)ZnL \xrightarrow[\text{slow}]{k_2} \text{apo-CA} + ZnL \xrightarrow[\text{fast}]{(n-1)L} \text{apo-CA} + ZnL_n$$

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TABLE I. Kinetic Parameters for the Reaction of Human Carbonic Anhydrase B[a] and Bovine Carbonic Anhydrase[b] Zn^{2+} Holoenzymes with Various Chelating Agents.

Chelating Agents	HCAB				BCA			
	K_{EML} (M^{-1})	k_2 (min^{-1})	K_2K_{EML} (M^{-1} min^{-1})	k_d[c] (min^{-1})	K_{EML} (M^{-1})	k_2 (min^{-1})	k_2K_{EML} (M^{-1} min^{-1})	k_d[c] (min^{-1})
2,6-PDA	30 ± 14	1.6 ± 0.5 × 10^{-1}	4.8		2.3×10^2	8.2×10^{-2}	18.9	
phen	35 ± 5	4.7 ± 5.0 × 10^{-3}	1.7 × 10^{-1}		3.8×10^2	2.1×10^{-2}	8.0×10^{-1}	
bipy			4.0 ± 0.5 × 10^{-2}	5×10^{-5}			5.0×10^{-2}	6×10^{-5}
EDTA			4.2 ± 0.3 × 10^{-3}	9×10^{-5}			0	2×10^{-5}

[a]Measured at 4 °C in 0.2 *M* acetate buffer (pH 5.0, μ =0.35). [b]The data for bovine carbonic anhydrase is taken from reference [8] and was obtained at 0 °C in 0.2 *M* acetate buffer (pH 5.0, μ = 0.33). [c]These 'spontaneous' dissociation constants may in fact include a contribution from any acetate dependent pathway present.

Fig. 1. Plot of $1/k_{obs}$ *versus* $1/[L]_o$ for the phen-HCAB system.

Assuming that k_2 is slow, and because L is in large excess, all steps after the first equilibrium are essentially irreversible. For this case,

$$k_{obs} = \frac{k_2 K_{EML}[L]_o}{1 + K_{EML}[L]_o} \quad (1)$$

where k_{obs} is the rate of appearance of the apoenzyme and $[L]_o$ is the initial ligand concentration.

The rate of zinc removal, as measured by the loss of enzyme activity, was determined over a range of concentrations for each chelating agent. For 2,6-PDA and phen the plots of k_{obs} against $[L]_o$ were curved whereas plot of $1/k_{obs}$ *versus* $1/[L]_o$ were linear, as required by eqn (1); the plot for the phen system is shown in Fig. 1. The experimental data for these two systems were fitted directly to (1) using a weighted non-linear least squares program to yield values for k_2 and K_{EML} (Table I). The individual values were found to vary somewhat from those obtained for the bovine enzyme by Kidani and Hirose [8] under similar conditions (Table I).

In contrast to the results for 2,6-PDA and phen, the plots of k_{obs} against $[L]_o$ for bipy and EDTA were linear over the ligand concentration range studied. Thus the $K_{EML}[L]_o$ term in eqn. (1) must be $\ll 1$ in these latter two cases indicating that there is no significant build up of the ternary complex (HCABZnL) during the reaction. For this situation eqn. (1) reduces to

$$k_{obs} = k_2 K_{EML}[L]_o \quad (2)$$

However since both bipy and EDTA are very slow to remove zinc from HCAB, the 'spontaneous' dissociation of zinc from HCAB also becomes significant·

$$HCABZn \xrightarrow{k_d} HCAB + Zn \xrightarrow[nL]{fast} HCAB + ZnL_n$$

For this situation, k_{obs} will be given by

$$k_{obs} = k_d + k_2 K_{EML}[L]_o \quad (3)$$

and k_2K_{EML} may be obtained directly from a plot of k_{obs} *versus* [L] (see Table I).

The mechanism of dissociation of zinc from HCAB in the presence of bipy appears similar to that of the bovine enzyme studied by Kidani and Hirose [8]. However, for the EDTA case, significant differences between the human and bovine enzymes were observed. For the human enzyme, as discussed above, two pathways appear to operate for production of the apoenzyme. The first is a first-order process in which EDTA sequesters free zinc following its dissociation from the holo-enzyme whereas the other is a second-order process involving formation of a ternary complex of zinc, protein and EDTA. The first-order mechanism appears to be solely operative in the bovine carbonic anhydrase-EDTA system under the conditions used [8]. With this exception, the mechanism of dissociation of the human enzyme in the presence of the other chelating agents thus appears generally similar to that for bovine carbonic anhydrase even though individual rate and equilibrium constants vary somewhat between the systems. Such variations are most readily rationalised in terms of minor differences in the active sites of these two forms of the enzyme [5].

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