^{81}Br-NMR STUDIES OF CARBONIC ANHYDRASE*

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Summary

^{81}Br nmr measurements have been made on high (bovine; BCA) and low (human-B; HCAB) specific activity forms of carbonic anhydrase and on a chemically modified form of the human enzyme (carboxyamidomethyl; CAM-HCAB). The high specific activity form of the enzyme, BCA, exhibits a ^{81}Br line broadening which is determined by the lifetime of Br^- bound to the zinc ion of the enzyme. The low specific activity form of the enzyme, HCAB, under similar conditions of concentration, pH, etc., does not exhibit a ^{81}Br nmr line broadening. Cl^-/Br^- competitive binding studies, using ^{35}Cl nmr, suggests that the failure to observe ^{81}Br broadening is due to an increase in the lifetime of a zinc bound Br^-. An increase in this lifetime by a factor of 10-100 over that exhibited by BCA is sufficient to abolish the line broadening. A modified form of HCAB, CAM-HCAB, does, however, exhibit a ^{81}Br nmr line broadening. Estimates of the lifetime of zinc bound Br^-, τ_M, are 4×10^{-7} sec. for CAM-HCAB at pH 8 and 1×10^{-7} sec. for BCA at pH 7. The lifetime for Br^- bound to HCAB is estimated to be $\geq 10^{-6}$ sec.

Chloride nmr probe studies of the various forms of carbonic anhydrase have shown that Cl^- can bind directly to the zinc ion of the enzyme (1,2). Furthermore, differences in ^{35}Cl relaxation data reflect differences in environments at the active sites of the high and low specific activity forms of the enzyme. For example, Cl^- binding studies of human carbonic anhydrase B (HCAB) and C (HCAC), low and high activity forms of the enzyme, reveal that the pK_h for the zinc ion in the two isoenzymes, extrapolated to zero Cl^-, is 8.7 and 7.0 respectively (3).

A temperature study of the Cl^- line broadening in the presence of HCAB

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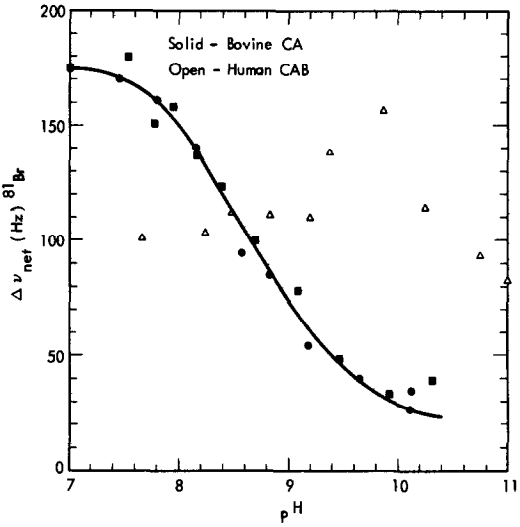

Fig. 1 Net line width, $\Delta\nu_{net}$, i.e., $\Delta\nu_{obsd} - \Delta\nu_{Br^-}$, vs. pH for solutions of bovine carbonic anhydrase (solid symbols), 0.8 mg/ml, and human carbonic anhydrase B (open symbols), 4 mg/ml, in 0.5 M NaBr. The pK_a for the bovine enzyme is 8.6.

indicated that the broadening is determined by the lifetime of Cl^- bound to the zinc ion of the enzyme (3). Further comparisons of line broadening using both ^{35}Cl and ^{37}Cl isotopes also indicates that the exchange rate is the dominating factor in the line broadening (4).

^{81}Br nmr studies of CA should complement the Cl^- studies. We have previously shown that Br^- and Cl^- compete for the Zn site of the bovine enzyme (3). Data in the literature indicate that although the ^{81}Br line width increases in the presence of HCAB, there is no change in the broadening upon the addition of a sulfonamide inhibitor (5). These observations appear to be in conflict with the Cl^- studies (1,2).

In examining this point further, we observe that ^{81}Br nmr line broadenings in the presence of BCA do complement our Cl^- results. The $^{81}Br^-$ line broadening observed is pH dependent (Figure 1) and this pH behavior is quite similar to that of Cl^- binding observed with ^{35}Cl nmr. Furthermore, the $^{81}Br^-$ line broadening is reduced in the presence of cyanide and acetazolamide, a powerful sulfonamide inhibitor (4). Titrations with these inhibitors yield

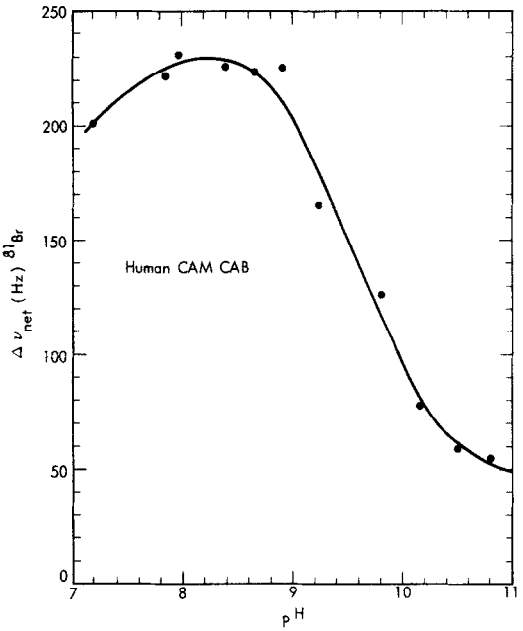

Fig. 2 Net line width, $\Delta\nu_{net}$, i.e., $\Delta\nu_{obsd} - \Delta\nu_{Br^-}$, vs. pH for a solution of modified (carboxyamidomethyl) human carbonic anhydrase B, 3.4 mg/ml, in 0.5 M NaBr. The pK_a is 9.6.

results identical to those obtained by ^{35}Cl nmr, i.e., the addition of one equivalent of inhibitor reduces the line width to that observed at high pH.

Since the exchange rate of Cl^- is the determining factor in the line broadenings of Cl^- in the presence of BCA, it is reasonable to expect that the same mechanism would apply to Br^-. If this were the case the line width ratios of $^{79}Br/^{81}Br$ isotopes, measured in the same solution of CA, would be 1.0. If the fast exchange limit pertained, the ratio of the line widths measured in Hz would be 1.39; in the ratio of the square of the quadrupole moments. We observe a ratio of 1.0 for the bovine system. The rate of Br^- exchange therefore determines the line broadening for BCA.

HCAB, however, under identical conditions of concentration, pH, etc. does not affect the ^{81}Br nmr linewidth appreciably. The line broadening observed for a solution of HCAB containing five times the amount of BCA is also shown in Figure 1. At present this nondescript broadening observed in this more concentrated solution must be ascribed to a variety of effects

including viscosity, nonspecific halogen binding, and perhaps a small amount of broadening due to the zinc ion. Examination of ^{35}Cl line broadenings produced by HCAB in the presence of varying amounts of Br^-, however, indicates competition between Cl^- and Br^- for the zinc site. We thus suggest that for HCAB the exchange rate of zinc bound Br^- is sufficiently slow that no effect is observed on the free Br^- line width. A decrease in the rate of exchange by a factor of 10-100 over that estimated for the bovine enzyme would be sufficient to explain our results.

We have also examined the carboxamidomethyl form of the human B enzyme, CAM-HCAB. This derivative of HCAB is prepared by the reaction of iodoacetamide with a single histidyl residue (His 204) located near the active site (6). It possesses 3% of the CO_2 hydrating activity and 30% of the esterase activity of the native enzyme (7). Chloride nmr studies of this derivative yield information quite similar to that reported for the native enzyme (4). In contrast to the ^{81}Br studies of the native enzyme, HCAB, however, the modified enzyme exhibits a ^{81}Br broadening quite similar to that observed for the bovine enzyme (Figure 2). The modification of His-204 with an uncharged carboxyamidomethyl group thus results in a decrease in the lifetime of bromide ion bound to the zinc ion.

The discussion presented elsewhere (3) for ^{35}Cl broadening in the presence of BCA can be extended to include the Br^- results. The relationship between line width and exchange lifetime is given by $\Delta\nu = f/\pi(T_{2M} + \tau_M)$ where τ_M is the exchange lifetime of bound bromide, T_{2M} is the transverse relaxation time, and f is the fraction of total bromide which is bound to the zinc ion of carbonic anhydrase.

The slow-exchange limit, i.e., $\tau_M \gg T_{2M}$, pertains to the bromide results presented here and the lifetime of zinc bound bromide ion can be estimated with a knowledge of f. The fraction of bound bromide, f, can be calculated from the value of K_I obtained from chloride- bromide competitive experiments (4). These values are 32 mM for BCA and 32 mM for CAM-HCAB at pH 7.5 and 16

mM for HCAB at pH 8. The f values are, however, not particularly sensitive to K_I values this small. Calculated values for τ_M are 4×10^{-7} sec. at pH 8 and 1×10^{-7} sec. at pH 7 for CAM-HCAB and BCA respectively. These pH values correspond to the maximum broadenings. These times are approximately an order of magnitude less than that observed for aqueous zinc bromide solutions (8). Aqueous zinc ions are, however, octahedral whereas the zinc in carbonic anhydrase is in a slightly distorted tetrahedral environment (6). The lifetime of Br^- bound to zinc in HCAB is estimated to be $\geq 10^{-6}$ sec.

The Br^- exchange mechanism is either competitive or dissociative, i.e., $E\text{-}Zn\text{-}Br^- + Br^{-*} \rightleftarrows E\text{-}Zn\text{-}Br^{-*} + Br^-$ or $E\text{-}Zn\text{-}Br^- \rightleftarrows E\text{-}Zn + Br^-$. A line width measurement as a function of total Br^- concentration can distinguish between these two possibilities (9). Measurements on the bovine enzyme indicate that the dissociative mechanism is operative, i.e. a straight line plot of $\Delta\nu^{-1}_{net}$ vs. $[Br^-]$ is obtained for BCA in the slow exchange limit. This result is in agreement with similar anion nmr studies of the cobalt substituted enzyme (10).

These studies yield additional support for the conclusion that the zinc ion environment of the high and low activity forms of carbonic anhydrase must be different (2). Furthermore, modification of histidine residue - 204 in HCAB by an uncharged carboxyamidomethyl group is reflected in a decrease in lifetime for zinc bound bromide ion. Other properties of the active site of HCAB are also perturbed by the modification and these are discussed elsewhere (7).

Materials and Methods

A carboxamidomethyl derivative of human erythocyte carbonic anhydrase B (CAM-HCAB) was prepared and purified by affinity chromatography (7). All measurements were carried out at a fixed field value of 14,098 gauss with a frequency swept spectrometer. The ^{81}Br measurements were made at 12.206 MHz, ^{79}Br at 15.036 MHz, and ^{35}Cl at 5.878 MHz. All titrations, including pH measurements, were carried out at 32°C.

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