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Low and High pH Form of Cadmium Carbonic Anhydrase Determined by Nuclear Quadrupole Interaction†

Rogert Bauer,* Peter Limkilde, and Jack T. Johansen

ABSTRACT: The pH dependence of the nuclear quadrupole interaction between the excited 247-keV state in ^{111}Cd bound to the active site in human carbonic anhydrase B and the nearest protein surroundings has been studied by means of the nuclear spectroscopic technique of perturbed angular correlation of γ rays. The enzyme has been studied in the pH region 5.6-11.0 at 22 and −196°C. The results show that the Cd enzyme changes from one form at low pH to another form at high pH both at 22 and −196°C. The pK of the transition is 8.9 ± 0.2 at −196°C and close to 9 at 22°C. Parallel to this transformation, the esterase activity of the Cd enzyme for the hydration of *p*-nitrophenyl acetate exhibits a pH dependency with a pK of 9.1 ± 0.2. The sulfonamide inhibitor acetazolamide completely inhibits this activity of the Cd enzyme. The quadrupole interaction parameters for the Cd enzyme are not significantly different at −196°C from those obtained at 22°C. A measurement at 0°C pH 5.7 shows, however, a form different from those at 22°C pH 5.6 and −196°C pH 5.7. The change in the quadrupole interaction with pH is, in a simple model, consistent with an ionization of a metal-bound water molecule.

Carbonic anhydrase is a zinc metalloenzyme which contains one tightly bound zinc ion essential for activity. The enzyme catalyses the reversible hydration of carbon dioxide, as well as the hydrolysis of esters and the hydration of aldehydes (for an extensive review see Lindskog et al., 1971). X-ray diffraction studies of human carbonic anhydrase B and C have established that the enzyme is almost spherical in shape with a radius of about 20 Å and that the Zn^{2+} ion is located at the bottom of the 12-15 Å deep active site cavity with three histidine residues and possibly a solvent molecule as its ligands (Kannan et al., 1975; Liljas et al., 1972). The essential Zn^{2+} can be replaced with other divalent metals, e.g., Co^{2+}, Mn^{2+}, Cd^{2+}, and Cu^{2+}. The Co and Mn enzymes are the only derivatives which so far have been shown to exhibit substantial esterase and CO_2 hydration activity (Lindskog et al., 1971; Lanir et al., 1975). However, as demonstrated in this paper, the Cd enzyme also exhibits activities but the pH-activity profile is shifted to higher pH value as compared to the Zn and Co derivatives.

Although numerous techniques have been employed to investigate the structure-function relationships of this enzyme (Lindskog et al., 1971), the mechanism of the enzymatic reaction has not been established with certainty (Lindskog and Coleman, 1973). It has been shown that a basic form of a group closely linked to the metal ion and having a pK near 7 is involved in catalysis. Spectroscopic changes with pH for various metal derivatives of carbonic

† From the Niels Bohr Institute, University of Copenhagen, Copenhagen, Denmark, and the Carlsberg Laboratory, Chemical Department, Copenhagen, Denmark. *Received August 15, 1975.* This work was supported by Grants No. 511-3539 and 511-5062 from the National Research Council of Denmark.

* To whom to address correspondence at the Niels Bohr Institute.

anhydrase (Lanir et al., 1973, 1975; Koenig and Brown, 1972; Fabry et al., 1970; Lindskog and Nyman, 1964; Taylor and Coleman, 1971; Fitzgerald and Chasteen, 1974) have shown the presence of low and high pH forms of the enzyme and shown that the transformation at the metal site is characterized by a pK similar to that controlling the enzymatic activity. It has been proposed that the functional group responsible for the transformation is a metal-bound water molecule which is titrated to a metal-bound hydroxide group at alkaline pH. The alkaline form of the enzyme is then the active species (Lindskog and Coleman, 1973). Recent high resolution proton NMR results where the histidine group of the enzyme is observed strongly support this proposal (Campbell et al., 1974). However, from the pH dependence of solvent proton relaxation in the presence of Co^{2+} and Mn^{2+} carbonic anhydrase (Fabry et al., 1970; Lanir et al., 1973, 1975; Koenig and Brown, 1972) it was suggested that a water molecule rather than a hydroxide ion is directly linked to the metal at high pH, while no water molecule is bound at low pH values. This suggests that the mechanism involves an ionizable basic amino acid side chain, which has been proposed to be a histidyl residue (Pocker and Stone, 1967, 1968; Pesando, 1975; Appleton and Sarkar, 1974). In this paper we have used the Cd^{2+} ion as a site-specific probe in conjunction with the method of perturbed angular correlation of γ rays (Bauer et al., 1974) to investigate the pH-dependent environment of the metal-binding site of carbonic anhydrase. The results are consistent with the hydroxide mechanism (Lindskog and Coleman, 1973) but the pK for the structural transition from the low to high pH form for the Cd^{2+} derivatives is shifted to about 9. Furthermore it is shown that the activity toward p-nitrophenyl acetate is similarly shifted to higher pH values with a similar pK.

In perturbed angular correlation of γ rays, an excited metal nucleus functions as a spectroscopic probe, and for the application of PAC[1] to Zn enzymes the most suitable metal is Cd. The result of a PAC measurement is a time spectrum, showing the development in time of the hyperfine interaction frequencies. This means that both static and dynamic behavior can be derived from such an experiment (Frauenfelder and Steffen, 1965). In a water solution at room temperature the rotational tumbling of molecules with molecular weight smaller than 3×10^5 occurs at such a high rate that the main part of the structure in the PAC spectrum is averaged out. This effect has been observed in a PAC experiment on Cd carbonic anhydrase dissolved in water (Meares et al., 1969). In the present work the rotational diffusion of carbonic anhydrase (with a molecular weight of 3×10^4) has been slowed down by using sucrose to increase viscosity or by freezing the enzyme solution. This has enabled us to determine the static quadrupole interaction between the Cd nucleus at the active site and its environment, and to study the pH dependence of this interaction.

With the introduction of PAC to the study of local protein structure (Bauer et al., 1974) we have extended the group of spectroscopic metal probes to Cd which is chemically close to Zn. Finally one should stress the great advantage of PAC measurements since they can be performed on metalloenzymes both in solution and in the crystalline state.

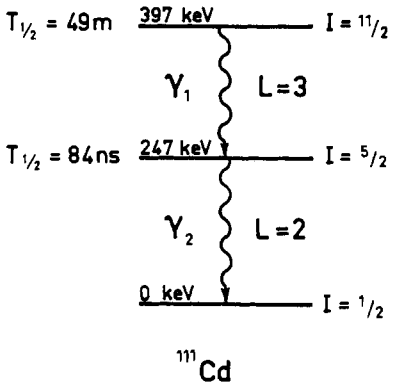

FIGURE 1: Decay scheme for the 397-keV isomer in ^{111}Cd.

Theory of PAC and Nuclear Quadrupole Interaction. In PAC experiments the angular correlation of two γ rays in succession is detected (Figure 1). The spin of the three nuclear levels involved and the angular momentum L of the two γ rays determine the angular correlation function of γ_1 with respect to γ_2. If, in addition, the time difference t between γ_1 and γ_2 is measured, the correlation function can be expressed

$$W(\theta,t) = e^{-t/\tau}W(\theta) \quad (1)$$

where $W(\theta)$ is the angular correlation at $t = 0$, θ is the angle between the two γ rays, and τ is the lifetime of the intermediate state. The angular correlation might now be perturbed if the intermediate level interacts with the surroundings, i.e., the level is split by hyperfine interaction. Such an interaction reorients the population of the magnetic substates as function of time and perturbs $W(\theta,t)$ (Frauenfelder and Steffens, 1965).

For the relevant cascade in ^{111}Cd (Figure 1) the angular correlation can be written as follows

$$W(\theta,t) = e^{-t/\tau}(1 + A(3/2\cos^2\theta - 1)G(t,\Delta E_{hf})) \quad (2)$$

where A is the amplitude of the unperturbed correlation and $G(t\Delta E_{hf})$ is a function describing the influence from the hyperfine splitting ΔE_{hf} in the case of randomly distributed molecules such as in a liquid or in a frozen solution.

The energy of the electrostatic interaction between the nucleus and the surroundings may be written

$$E_{hf} = \int\int \frac{\rho(\vec{r}_n)\rho(\vec{r}_s)}{|\vec{r}_n - \vec{r}_s|} dV_n dV_s$$

and multipole expanded in $1/|\vec{r}_n - \vec{r}_s|$ as

$$E_{hf} = \frac{4\pi}{2l+1}\sum_{lm}\int \rho(\vec{r}_n) r_n{}^l Y^*_{lm}(\theta_n,\phi_n) dV_n \times \int \rho(\vec{r}_s) r_s{}^{-l-1} Y_{lm}(\theta_s,\phi_s) dV_s \quad (3)$$

where θ, ϕ, and r are polar coordinates of either the nuclear charge distribution $\rho(\vec{r}_n)$ or the charge distribution of the surroundings $\rho(\vec{r}_s)$, and $Y_{lm}(\theta,\phi)$ is the spherical harmonics. Since the $l = 0$ term is isotropic, it is unobservable in an angular correlation experiment. Because the nuclear charge distribution has inversion symmetry, only terms with even l will influence the angular distribution. Due to the $r_s{}^{-l-1}$ dependency only the quadrupole term ($l = 2$) is significant. This term can be written

[1] Abbreviations used are: PAC, perturbed angular correlation of γ rays; TAC, time-to-amplitude converter; HCAB, human carbonic anhydrase B; HCAC, human carbonic anhydrase C; Mes, 2-[N-morpholino]ethanesulfonic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane sulfate; Caps, cyclohexylaminopropane sulfonic acid.

$$E_2 = \frac{4\pi}{5}\sum_q V_q^2 A_q^2 Q$$

where Q is the nuclear quadrupole moment, A_q^2 is known intermediate spin dependent constants, and V_q^2 is equal to the $l = 2$ part of the second integral in (3). V_q^2 can be expressed in terms of the electric field at the nucleus as

$$V_{20}^2 = V_{zz}/2$$
$$V_{2\pm1}^2 = \mp(V_{xz} \pm iV_{yz})\sqrt{6}$$
$$V_{2\pm2}^2 = (V_{xx} - V_{yy} \pm 2iV_{xy})/2\sqrt{6}$$

where $V_{\alpha\beta} = (\partial/\partial\beta E_\alpha)_{r_s=0}$, $\alpha,\beta = x$, y, or z, and $\vec{E}$ is the electric field produced by the charge distribution $\rho(\vec{r}_s)$. Since the molecules in all experiments described here are randomly oriented, the coordinate system can be chosen freely. Therefore the principal system which is diagonal, i.e., $V_{\alpha\beta} = 0$ when $\alpha \neq \beta$, and $|V_{zz}| \geq |V_{yy}| \geq |V_{xx}|$ is used. In addition $V_{xx} + V_{yy} + V_{zz} = 0$, because the nonisotropic part of the electronic charge density at the nucleus is negligible. This means that the electric field gradient can be specified by the two parameters V_{zz} and $\eta = (V_{yy} - V_{xx})/(V_{xx} + V_{yy})$, where $0 \leq \eta \leq 1$. However, it should be noted that only the numerical value of V_{zz} can be determined from a PAC experiment. In the cases where measurements are performed with single crystals one gets in addition the three directions of the principal system axis. The form of $G(t,\Delta E_{hf})$ for $I = 5/2$ is

$$G(t,\Delta E_{hf}) = \sum_{p=0}^{3} a_p(\eta)\cos(b_p(\eta)|V_{zz}|t) \quad (4)$$

Examples of this function can be found in Frauenfelder and Steffens (1965) and Bauer et al. (1974).

Time-dependent quadrupole interaction requires a modification of eq 4. The influence of time dependent interaction is in general strongest for $\omega_0\tau \simeq 1$ where τ_c is the correlation time for the time dependent process and $\omega_0 = 3eQ|V_{zz}|/\hbar 2I(2I-1)$. One time-dependent process which is always present in solution is the rotational diffusion of the molecules, and for such a process the effect on the angular correlation in the case $\omega_0\tau_c \simeq 1$ will be a rapid damping of the anisotropy as function of time. In the region where $\omega_0\tau_c \ll 1$ the following expression for G is given (Frauenfelder and Steffens, 1965):

$$G(t,\Delta E_{hf},\tau_c) = e^{-\lambda t} \quad (5)$$

where $\lambda \propto \langle V_{zz}^2\rangle\tau_c$. At the other extreme where $\omega_0\tau_c \gg 1$ the form of G is (Marshall and Meares, 1972):

$$G(t,\Delta E_{hf},\tau_c) = e^{-t/\tau_c}G(t,\Delta E_{hf}) \quad (6)$$

Therefore, only in the case where $\omega_0\tau_c \gg 1$ is it possible to determine both $|V_{zz}|$, η, and τ_c. For $\omega_0\tau_c \ll 1$ only the product $\langle V_{zz}^2\rangle\tau_c$ can be determined. The rotational diffusion time τ_c can be estimated by use of the Stoke-Einstein formula

$$\tau_c = 4\pi a^3\tilde{\eta}/3kT \quad (7)$$

where a is the radius of the molecule, $\tilde{\eta}$ is the viscosity of the solvent, k is Boltzmanns constant, and T is the absolute temperature.

Finally, the form of G might change if several different interactions take place simultaneously, i.e., the metal has several binding sites or the metal enzyme complex exists in several forms. This results in

$$\overline{G(t)} = \sum_n G(t,\omega_n)P(n) \quad (8)$$

where $P(n)$ represents the fraction of each form. If the distribution function $P(n)$ is of a Gaussian form then

$$\overline{G(t)} = \int_0^\infty G(t,\omega)e^{-(\omega-\omega_0)^2/2\sigma^2}d\omega \Big/ \int_0^\infty e^{-(\omega-\omega_0)^2/2\sigma^2}d\omega \quad (9)$$

where ω_0 is the mean frequency and σ is the variance.

The two parameters V_{zz} and η can be expressed in terms of the molecular charge distribution surrounding the nucleus as:

$$V_{zz} = -e\int \psi^*(\vec{r}_e)(3\cos^2\theta_e - 1)/r_e^3 \times \psi(\vec{r}_e)\,dV_e + \sum_i e(3\cos^2\theta_i - 1)/r_i^3 \quad (10)$$

and

$$V_{xx} - V_{yy} = \eta V_{zz} = e\int\psi^*(\vec{r}_e) \times 3\sin^2\theta_e\cos 2\phi_e/r_e^3\psi(\vec{r}_e)\,dV_e - \sum_i e3\sin^2\theta_i\cos 2\phi_i/r_i^3 \quad (11)$$

where (r_e, θ_e, ϕ_e) and (r_i, θ_i, ϕ_i) are the polar coordinates of the position of the electron and the nuclei, where the origin is the Cd nucleus, ψ is the electronic wave function, and e is the unit charge.

In general the main contribution to the field gradient arises from the electrons at the central atom and the electrons and nuclei of the ligands, because of the rapidly decreasing function r^{-3}.

For the central atom strong field gradients may be present from singly occupied d and p orbitals, whereas s orbitals give no contribution due to the spherical symmetry.

In the case of Cd this means that the electrons of neither the metal atom nor the metal ion will produce any field gradient at the nucleus. It is therefore apparent that in Cd complexes the field gradient must be produced and/or induced by the ligands.

A single 5p orbital has a field gradient of the magnitude

$$|V_{zz}| = 1.1 \times 10^{16}\ \text{esu},$$

as measured in atomic ^{115}In (Lucken, 1969). This value for $|V_{zz}|$ gives for the characteristic quadrupole frequency of the 5/2 level in ^{111}Cd using the recently determined $Q = 0.77 \pm 0.12 \times 10^{-24}$ cm^2 (Raghavan et al., 1973)

$$\omega_0 = \frac{3eQV_{zz}}{2I(2I-1)\hbar} = 6.0 \times 10^2\ \text{MHz} \quad (12)$$

In the case of an ionic ligand of one unit charge in a typical bonding distance of 2 Å we get if we approximate the ion with a point charge

$$|V_{zz}| = 3.6 \times 10^{15}\ \text{esu}$$

or

$$\omega_0 = 2.0 \times 10^2\ \text{MHz} \quad (13)$$

The value has been corrected for the polarization of the closed shell electrons of Cd (Feiock and Johnson, 1969).

In the case of a covalent bond in a Cd complex one would expect that a large fraction of the bond consists of ligand-directed σ orbitals. This expectation makes it reasonable to apply the point charge model in order to derive molecular

Table I: Electric Field Gradient for Various Point Charge Complexes with One Charge per Ligand in 2-Å Distance.

No. of Ligands	Type	V_{zz} (esu $\times 10^{-15}$)	η	ω_0 MHz(^{111}Cd)	Angle in degrees Between Ligands
1	Axial	3.6	0	200	
2	Axial	7.2	0	400	180
3	Plane trigonal	5.4	0	300	120
3	Trigonal pyramid	0		0	90
4	Square planar	7.2	0	400	90,180
4	Tetrahedral	0		0	109.5
5	Square pyramid	0		0	80.4,114.1
6	Octahedral	0		0	90,180

Table II: Experimental Conditions of the PAC Experiments.

Expt No.	Temp (°C)	pH	HCAB Concn (μM)	Buffer	Buffer Concn (M)	Cl^- Concn (M)[a]	Sucrose Concn (M)
1	22	5.6	6	Mes	0.02	0.02	1.83
2	22	5.6	20	Mes	0.02	—	1.83
3	22	9.1	20	Tris	0.02	—	1.83
4	22	11.0	20	Tris	0.001	—	1.83
5	0	5.7	6	Mes	0.02	0.02	1.83
6	0	5.7	6	Mes	0.02	—	1.83
7	−196	5.7	30	Mes	0.05	—	
8	−196	8.3	30	Hepes	0.05	—	
9	−196	9.1	100	Tris	0.05	—	
10	−196	9.9	100	Tris	0.05	—	

[a] A dash (—) means concentrations very much lower than 0.001 M.

information from the field gradient. In this model it is assumed that bonding occurs only via σ bonding. The strength of the field gradient from each ligand–metal bond is represented by that of a pseudo point charge on the ligand so that the total field gradient can be written as:

$$V_{zz} = \sum_i S_i(3\cos^2\theta_i - 1)/r_i^3 \qquad (14)$$

The parameter S_i then represents the formal charge or pseudo charge on the ith ligand and is defined so that

$$S_i(3\cos^2\theta_i - 1)/r_i^3$$

is equal to the field gradient produced by the ith molecular orbital at the nuclei. The field gradient for various symmetric point charge complexes are tabulated in Table I.

Materials and Methods

Human carbonic anhydrase B was isolated from hemolyzed erythrocytes by affinity chromatography (J. T. Johansen, manuscript in preparation). The apoenzyme was prepared essentially as described by Coleman (1965) by dialysis of the enzyme against 0.01 M 1,10-phenanthroline for 2 weeks. Stock solutions of apoenzyme (40–70 mg/ml) were obtained by dialyzing the apoenzyme for three successive times against either 0.05 M Mes,[1] 0.05 M Hepes, or 0.05 M Tris buffer of the desired pH and then concentrated by ultrafiltration. The protein concentration was determined from the absorbance at 280 nm using a molar absorptivity of $4.69 \times 10^4\ M^{-1}\ cm^{-1}$, (Nyman and Lindskog, 1964). The residual zinc content of the apoenzyme was estimated from activity measurements to be less than 5%. When completely metal-free apoenzyme was necessary, the apoenzyme was passed over a metal-free affinity column, the same as used for isolation of enzyme, which only binds the metalloenzyme. By this procedure an apoenzyme containing less than 1% zinc could be obtained (J. T. Johansen, manuscript in preparation). The enzymatic activity was determined using p-nitrophenyl acetate (1 mM) as a substrate by following the change in absorbance at 348 nm (Armstrong et al., 1966). The observed rate was corrected at each pH for the rate of ester hydrolysis in the absence of enzyme. Furthermore, the apoenzyme was used as a control. The Zn^{2+}, Cd^{2+}, and Ni^{2+} derivatives were prepared by addition of a threefold excess of the desired metal, followed by gel filtration on Sephadex G-25 to remove the excess metal. All buffers used were freed of trace metal contamination by extraction with 0.1% dithizone in carbon tetrachloride (Thiers, 1957). Glassware and cuvettes were cleaned by soaking in 1:1 nitric and sulfuric acids, followed by rinsing in metal-free distilled water.

The absorption spectra were recorded on a Beckmann Acta III or a Cary 15 spectrometer. Acids and hydroxides were prepared by dissolving or diluting analytical grade chemicals in double glass distilled water. Sucrose solutions were prepared by dissolving anhydrous crystalline sucrose in 0.05 M Mes or Tris buffers. At pH values above 10 the sucrose itself functions as the buffer, and the pH was adjusted with NaOH.

The ^{111}Cd radioactive atoms were produced by bombardment of metallic Pd with α particles at 20 MeV from the Niels Bohr Institute's Tandem Van de Graaff accelerator, thereby populating the 397 keV isomeric state in ^{111}Cd, which has a half-life of 49 min. The Pd target was isotopically enriched with ^{108}Pd to more than 94% and had a total weight of 2–4 mg while the target thickness was 40–50 mg/cm^2.

After bombardment, the Pd metal was dissolved in hot Aqua Regia, evaporated to dryness, and redissolved in 0.1 N HCl. The Cd atoms were isolated from the Pd by a dithizone procedure (De Voe and Meinke, 1959), which gives a carrier free solution of Cd^{2+} in 0.1 N HCl. The pH was adjusted with NaOH and one of the buffers mentioned above. The sample had at this stage a volume of 1–1.5 ccm, with a buffer strength of 0.005 M and NaCl concentration of 0.1 M. The pH of the sample was in the interval 5.5–9.0. The ^{111}Cd enzyme was obtained by adding 50–500 μl of the stock apoenzyme solution in the desired buffer to the total Cd^{2+} sample. The binding was allowed to proceed for at least 5 min. In a separate tracer experiment it was found that the ratio of Pd atoms to apoenzyme molecules was less than 2%. Before the measurements all samples except 1 and 5 (see Table II) were passed over a Sephadex G-25 column (1.4 × 15 cm) in order to separate Cd bound to the enzyme from free Cd^{2+} and to obtain samples free from Cl^-. The Cd atoms were traced by their radioactivity working with a NaI scintillator. In the experiments at 22 and 0°C 1 ml of the Cd-HCAB solution was added to a sucrose solution giving the final volume of 5 ml of 50.8% weight sucrose and with a buffer concentration of about 0.02 M. At pH values above 10 the sucrose solution was titrated with NaOH, and final sodium sucrose concentration was about 0.2 M.

The sample temperature was stabilized by means of a peltier element. The temperature was measured with a plat-

Table III: Quadrupole Interaction Parameters Determined in the Separate PAC Experiments on ^{111}Cd-HCAB.[a]

Expt No.	Temp (°C)	pH	ω_{01} (MHz)	ω_{02} (MHz)	% ω_{02}	η_1	η_2	δ	τ_r (nsec)
1	22	5.6	99 ± 10	(150)	14 ± 33	0.69 ± 0.18	(0.00)	0.14 ± 0.33	$83 \pm \frac{110}{40}$
2	22	5.6	90 ± 9	(81)	29 ± 30	0.98 ± 0.34	(0.22)	$0.18 \pm \frac{0.12}{0.08}$	$78 + \frac{89}{35}$
3	22	9.1	71 ± 14	199 ± 27	67 ± 13	0.74 ± 0.52	0.34 ± 0.21	$0.16 \pm \frac{0.20}{0.08}$	$60 \pm \frac{37}{21}$
4	22	11.0	(88)	232 ± 27	92 ± 18	(0.00)	0.19 ± 0.19	$0.18 \pm \frac{0.14}{0.07}$	$144 + \frac{\infty}{95}$
5	0	5.7	79 ± 6	(144)	12 ± 34	0.73 ± 0.16	(0.00)	0.07 ± 0.10	$99 \pm \frac{118}{42}$
6	0	5.7	82 ± 8	(162)	22 ± 28	0.72 ± 0.24	(0.00)	0.19 ± 0.10	$135 \pm \frac{365}{68}$
7	−196	5.7	94 ± 8	(139)	20 ± 45	0.72 ± 0.16	(0.20)	0.18 ± 0.07	
8	−196	8.3	95 ± 4	226 ± 39	23 ± 12	0.71 ± 0.11	0.29 ± 0.30	0.10 ± 0.09	
9	−196	9.1	102 ± 11	274 ± 30	62 ± 13	0.78 ± 0.23	0.00 ± 0.31	$0.17 \pm \frac{0.13}{0.08}$	
10	−196	9.9	(112)	232 ± 24	89 ± 24	(0.47)	0.21 ± 0.18	$0.19 \pm \frac{0.12}{0.06}$	

[a] When a second interaction is not determined significantly the corresponding parameters are enclosed in parentheses.

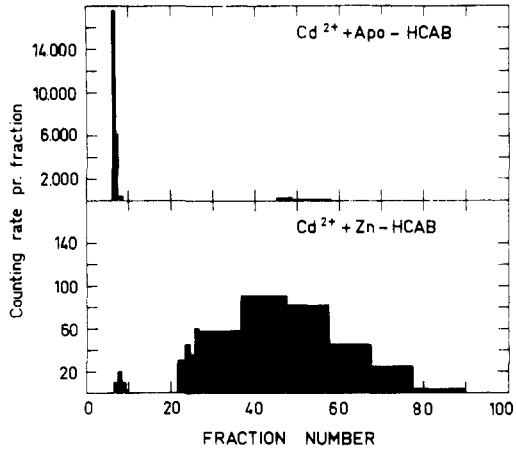

FIGURE 2: Elution curves for $^{111}Cd^{2+}$ in the presence of apo-HCAB and Zn-HCAB. The column was Sephadex G-25 fine equilibrated with 0.05 *M* Tris buffer at pH 8.5. The fraction size was 1.5 ml and the counting rate (cps) of the 247-keV γ rays from ^{111}Cd was recorded for each fraction.

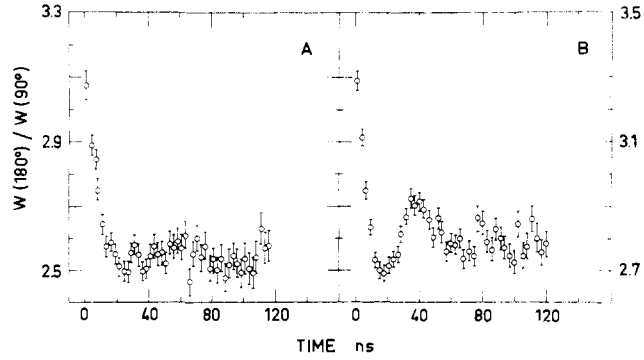

FIGURE 3: Time spectrum of ^{111}Cd bound to the active site in HCAB, pH 5.5, T = 22°C. (A) Without sucrose; (B) 50.8% (weight) sucrose.

inum thermistor covered with glass or ceramics which was placed in the middle of the sample. The uncertainty in the temperature determination was ±1°C. When cooling to −196°C the sample was immersed into liquid nitrogen. The temperature of the sample reached −196°C within 5 min.

The pH of the samples was determined immediately after the PAC measurements with a Radiometer pH 63 meter equipped with a GK 2301 B combination electrode. The pH values at −196°C are assumed to be identical with those measured on the sample at 0°C.

Four NaI detectors in fixed angles at 0, 90, 180, and 270° recorded the γ rays. Any registered γ ray in the detectors in 0 or 270° started the time-to-amplitude converter and any γ ray recorded in the counters at 90 or 180° stopped the TAC. The output pulsed from the TAC had a magnitude proportional with the time difference between the detected γ rays. An energy coincidence unit was used to reject all TAC signals that did not involve two γ rays at the right energies 150 and 247 keV. The energy signals from the separate counters were used to route the accepted TAC signals to appropriate sections of a multichannel analyzer. The time resolution of the NaI detectors at the present γ energies was 4–6 nsec.

Chi-square fits to the spectra allowing up to two different interactions to be present at the same time were made by using the function:

$$W(180°)/W(90°) = a + bG(\omega_{02},\eta_2,\tau_r,\delta) + c(G(\omega_{01},\eta_1,\tau_r,\delta) - G(\omega_{02},\eta_2,\tau_r,\delta))$$

where c/b and $1 - c/b$ are the percentages of the interactions ω_{01} and ω_{02}, respectively (Bauer et al., 1974; Meares et al., 1969). For the perturbation factor G we used eq 6 and allowed for a Gaussian distribution in ω_0 with variance $\sigma = \delta\omega_0$. The chi-square surface was searched for the absolute minimum in the parameters τ_r, ω_{01}, ω_{02}, η_1, η_2, δ, each time making a linear fit to the parameters a, b, and c. The uncertainty in the parameters was calculated from the curvature of the chi-square surface. Whenever the average curvature to the left of the minimum differs significantly from the curvature to the right both values for the uncertainty are stated.

Results

The Cd binding to the apoenzyme and Zn enzyme was investigated by gel-filtration experiments. The elution

Table IV: Quadrupole Interaction Parameters Summarized for ^{111}Cd-HCAB Determined by PAC Experiments.

−196°C		0°C	22°C	
Low pH Form	High pH Form	Low pH Form	Low pH Form	High pH Form
$\omega_0 = 95 \pm 4$ MHz	$\omega_0 = 244 \pm 18$ MHz	$\omega_0 = 81 \pm 5$ MHz	$\omega_0 = 95 \pm 7$ MHz	$\omega_0 = 232 \pm 27$ MHz
$\eta = 0.74 \pm 0.10$	$\eta = 0.17 \pm 0.16$	$\eta = 0.73 \pm 0.14$	$\eta = 0.68 \pm 0.18$	$\eta = 0.19 \pm 0.19$
$\delta = 0.16 \pm 0.05$		$\delta = 0.13 \pm 0.07$	$\delta = 0.18 \pm 0.07$	
$pK = 8.9 \pm 0.2$		$\tau_r = 99 \pm \frac{118}{42}$	$\tau_r = 81 \pm \frac{71}{27}$ nsec	
			$pK = 8.8 \pm 0.3$	

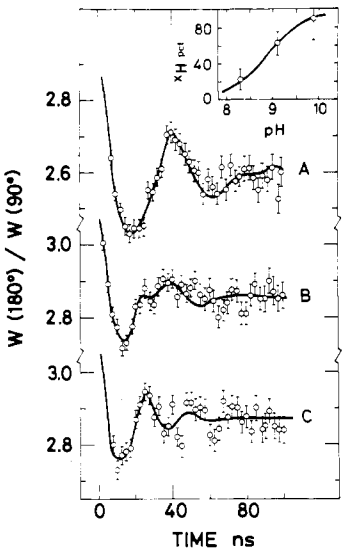

FIGURE 4: Time spectra of ^{111}Cd bound to the active site of HCAB in frozen solution at −196°C. (A) pH 8.3; (B) pH 9.1; and (C) pH 9.9. The fully drawn curve displays the theoretical function determined by least χ^2 fit to the experimental points. The insert shows the experimentally determined percentage of the high pH form, x_H, as function of pH, together with the theoretical curve of a single ionization with pK = 8.9.

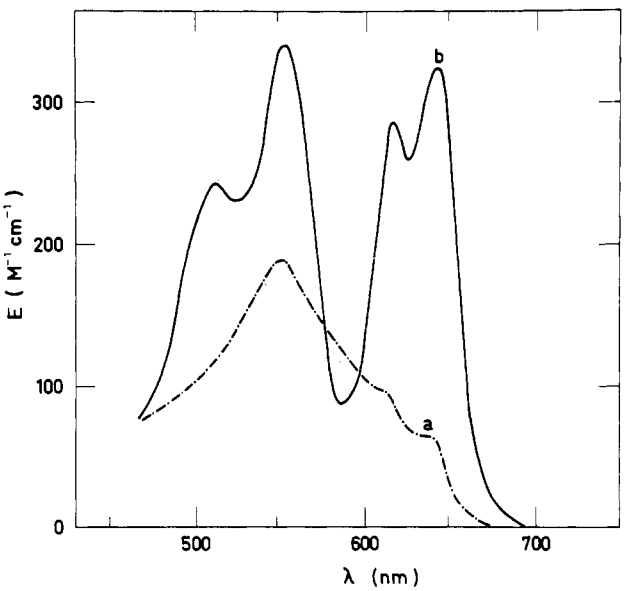

FIGURE 5: Visible absorption spectra of Co(II)-HCAB in sucrose. (a) pH 6.5, 46% sucrose; (b) pH 11.0, 48% sucrose.

curves for the Cd activity in the presence of apo-HCAB and Zn-HCAB with the column equilibrated with 0.05 *M* Tris at pH 8.4 are shown in Figure 2. From the integrated intensities in the peaks one finds that less than 2% of the Cd binds to the enzyme whereas more than 98% binds to the apoenzyme. Equivalent experiments carried out at pH 5.5 using 0.05 *M* Mes buffer gave the same result. In carrier free concentrations Cd^{2+} thus seems to have only one binding place in the enzyme and this site is identical with the Zn site.

The PAC results from ^{111}Cd bound to the active site in HCAB are presented in Table III which shows the parameters determined by least-chi-square fits to the experimentally recorded time spectra.

The experimental conditions of the PAC experiments are given in Table II. In two of the experiments a finite Cl^- concentration of 20 m*M* is present. This concentration is comparable to the K_i values of 15 and 51 m*M* reported for inhibition of Zn-HCAB with Cl^- (Whitney et al., 1967; Verpoorte et al., 1967). Nevertheless no difference has been observed in the PAC spectra taken at 0°C, pH 5.7 and 22°C, pH 5.6 with and without 20 m*M* Cl^-. This means probably that the K_i value of Cl^- inhibition of Cd-HCAB is greater than or equal to 20 m*M*.

Figure 3 shows the time spectra of Cd-HCAB in water-sucrose solution. The result demonstrates how the slowing down of the rotational tumbling of the molecules brings back some of the static structure in the time spectrum lacking in the pure water case. The viscosity of the sucrose solution was determined with an Ostwald type viscosimeter to be 18 ± 1 cP independent of pH up to 11.3.

The main result seen from Table III is that the enzyme changes from one form at low pH to another form at high pH. In Table IV the mean values of the quadrupole parameters determined for the low pH form and the high pH form are summarized together with the pK value for the transition from one form to the other.

In Figure 4 the time spectra of mixings of low pH form and high pH forms at −196°C are shown as examples of the experimental recordings. The transition from the low pH form to the high pH form can be fitted with a theoretical titration curve involving a single ionization with apparent pK of 8.9 ± 0.2 at −196°C and 8.8 ± 0.3 at 22°C.

Figure 5 displays the absorption spectra of the Co-HCAB measured in a 46% sucrose solution at pH 6.5 and 48% sucrose at pH 11.0. The spectra are seen to have the same characteristic form as those published for HCAB in water by Whitney (1970).

The pH dependence of the rate of hydrolysis of *p*-nitrophenyl acetate by Zn-, Cd-, and Ni-HCAB is shown in Figure 6. Sigmoid curves are obtained for both the Zn and Cd enzyme. Chi-square fits using a single ionization curve give apparent pK values of 7.3 ± 0.3 and 9.1 ± 0.2 and maximum activities of 41 ± 5 and 12 ± 2.5 min^{-1} for the Zn- and Cd-HCAB, respectively. Whereas the Cd enzyme at alkaline pH restores 29 ± 7% of the Zn-HCAB activity the

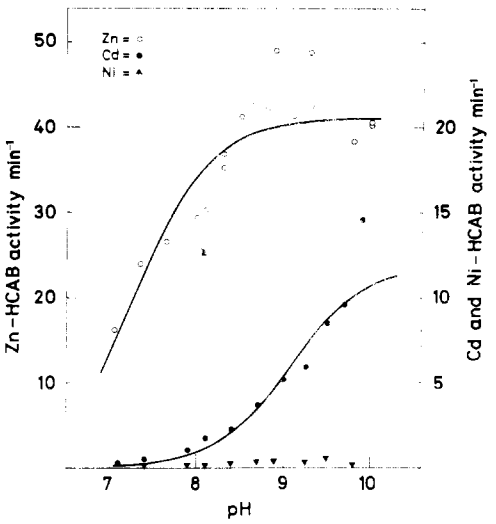

FIGURE 6: Zn (○), Cd (●), and Ni (▼) human carbonic anhydrases B catalyzed hydrolysis of *p*-nitrophenyl acetate as function of pH. The activity is presented as moles of substrate hydrolyzed per mole of enzyme per minute. The assay medium contained 1 m*M* *p*-nitrophenyl acetate, 1% v/v acetone, and 0.01 *M* buffer. Below pH 9.5 Tris was used as buffer; above this value the buffer was Caps. Protein concentrations were: Zn, pH 7-8, 4.3×10^{-6} *M* and above pH 8 1.1×10^{-6} *M*, Cd, 5.5×10^{-6} *M* and Ni, 5.5×10^{-6} *M*.

Ni derivative shows less than 1% of the Zn-HCAB activity independent of pH. Addition of acetazolamide at pH 9.6 completely inhibits the activity of the Cd enzyme with a binding constant of approximately 2×10^{-5} *M*.

The specific activity of Zn-HCAB was not affected by sucrose up to at least 50%.

Discussion

The present results demonstrate that ^{111}Cd is a highly relevant probe for studying the catalytical mechanism of carbonic anhydrase. The binding site for Cd is unique and identical with that of Zn (see Figure 2), the overall conformation of the Cd enzyme is the same as that of the Zn enzyme (Zavodsky et al., 1975), and the Cd enzyme is catalytically active at high pH values. The PAC spectrum of ^{111}Cd-HCAB is sensitive to the change caused by the activity linked ionization as well as changes induced by temperature or freezing.

The esterase activity measurements on the Zn and Cd enzyme and the Mn enzyme (Lanir et al., 1975) show a correlation between the p*K* for the activity and the ionic radius of the metal ion, namely that the p*K* increases when the ionic radius increases. A similar correlation between metal bond length and p*K* values has been found in a recent investigation of the p*K* values for a hydrolytic water molecule in different metal complexes (Coates et al., 1974). These two findings together seem then to support the proposed hydroxide mechanism for the catalytic activity of the enzyme (Lindskog and Coleman, 1973).

In all the experiments at 0 and 22°C a 50.8% sucrose solution has been used in order to slow the rotational diffusion of the enzymes. That this is necessary can be seen in Figure 3A where measurement on the enzyme in a water solution is present. It is seen that the spectrum consists only of a rapid damping of the structure. This will happen if $\omega\tau_R \simeq 1$, where τ_R is the rotational correlation time, as mentioned in the theoretical section. A similar experiment has been performed in a water solution with the same result (Meares et al., 1969). On the other hand, the addition of 50.8% sucrose results in a partially structured spectrum (Figure 3B) from which both the static quadrupole interaction and the rotational correlation time can be determined (Bauer et al., 1974). The rotational correlation time was calculated from eq 8 at 22°C in a water solution, to be 1.0×10^{-8} sec using an average molecular radius of 21.5 Å calculated from the dimension between extreme points for the enzyme (Kannan et al., 1975). As seen in Tables III and IV the frequencies in solution are close to 100 MHz thus giving $\omega\tau_R \simeq 1$. If we now use eq 7 for the sucrose solution we would get $\tau_R = 17 \times 10^{-8}$ sec at 22°C and $\tau_R = 50 \times 10^{-8}$ sec at 0°C. The observed rotational correlation times which are summarized in Table IV are significantly enhanced relative to the pure water solution, but at 0°C τ_R is more than a factor of two from the calculated value. This might be due to the fact that we have used a molecular radius corresponding to the extreme points of the enzyme. Furthermore, the experimental ratio

$$\tau_R(22°C)/\tau_R(0°C) = 1.2 \pm {}^{1.5}_{0.5}$$

is slightly inconsistent with the calculated value $\tau_R(22°C)/\tau_R(0°C) = 2.94$ and could be due to the fact that we have assumed the enzyme to be spherical.

The influence of sucrose on the active site structure of the enzyme seems to be negligible since the visible absorption spectrum of the Co(II) enzyme in a 46% sucrose solution at pH 6.5 and 48% sucrose at pH 11 is similar to those published for HCAB in pure water (Whitney, 1970) and the activity of the Zn enzyme toward *p*-nitrophenyl acetate at pH 8 is independent of the sucrose concentration.

The most striking result of the PAC measurement is the demonstration of two different forms for the Cd enzyme, one at low pH and another at high pH. The shift from one form to the other occurs at 22°C close to pH 9 in agreement with the p*K* deduced from the activity measurements. At −196°C the data can be well fitted by a single ionization curve (Figure 6) with a p*K* of 8.9 ± 0.2. The titration of the bovine Zn enzyme exhibits a second inflection point for the activity at pH 11.3 (Pocker and Watamori, 1973) but because of the change in p*K* with ionic radius and the lower value of the p*K* for the Cd activity, we believe that the observed transition corresponds to the first transition in the Zn enzyme. The inhibition of the Cd enzyme activity with acetazolamide supports this conclusion because acetazolamide only inhibits the activity at physiological pH for the Zn enzyme (Wells et al., 1975). The least-squares fit of the result at pH 9.1 T = 22°C yields a low pH form which is somewhat different from that determined in the other experiments. This might be because the rate of interconversion $1/\tau_E$ between the low and high pH form is about 10^8 sec^{-1} so that $\omega_0\tau_E \simeq 1$ whereby the use of eq 6 is not allowed. This possibility is currently under investigation (R. Bauer and P. Limkilde, to be published).

In an earlier investigation (Bauer et al., 1974) we have, at pH 6.5, observed a combination of the low pH form and an additional form with ω_0 = 135 MHz. This effect might be similar to the additional transition at pH 6.4 found in the absorption spectrum of Co(II)-HCAB (Whitney, 1970).

Since Cd usually acts more covalently than Zn it must be expected that sp^3 hybrid orbitals or equivalent orbitals are likely to be induced on Cd and that the field gradient from each bond therefore must be a significant fraction of hat of a single 5p electron. In the simple point charge model about three pseudo charges in 2 Å distance are required to match

the field gradient of a single 5p electron. Except for symmetries which have $V_{zz} = 0$, this would according to Table I give frequencies in the region 600–1200 MHz for the various symmetrical arrangements. The PAC result for the low pH form with a frequency of about 100 MHz indicates therefore that the geometry is close to a configuration for which $V_{zz} = 0$. With for example four ligands this means a geometry close to tetrahedral (Table I).

The three-dimensional structures of HCAC and HCAB have been determined at about pH 8.5 (Liljas et al., 1972; Kannan et al., 1975). In both isozymes the Zn atom is liganded to the protein by $N_{\epsilon 2}$ of His-96 and -94 and $N_{\delta 1}$ of His-119 in approximately tetrahedral angles. From the measured coordinates for the C form (K. K. Kannan and I. Vaara, private communication) the following angles were calculated: (N_{94}–Zn–N_{96}, N_{119}–Zn–N_{96}, N_{94}–Zn–N_{119}) = (116°, 119°, 104°) with an uncertainty of ±10°. This means that the symmetry is close to or identical with tetrahedral indicating the presence of a fourth ligand to complete the tetrahedral symmetry. However, the x-ray data do not permit a definite identification of this fourth ligand (K. K. Kannan and I. Vaara, private communication; Kannan et al., 1971; Liljas et al., 1972).

We will now use the point charge model in order to discuss possible explanations of the change in quadrupole interaction with pH. The discussion is based on the assumption that (1) the binding angles of the Cd-HCAB complex are close to those observed by the x-ray diffraction of the Zn-HCAC enzyme, (2) the fourth ligand binds in tetrahedral angles (within 10°), and (3) the binding angles do not change with pH. In the B form of the enzyme the three nitrogens liganded to Zn have distances close to 2.0 Å (K. K. Kannan, private communication). It seems from the x-ray analysis that the nitrogen Zn bonds are parallel with the corresponding imidazole plane with the exception of the N_{94}–Zn bond in both isozymes (K. K. Kannan and I. Vaara, private communication). We have therefore considered the strength of the field gradient from N_{94} and the fourth ligand to be different from those of the two other nitrogen bonds. In Figure 7, the effect of the field gradient strength from the Cd–N_{94} bond (S_3) and that from the fourth ligand bond (S_4) on η and V_{zz} are shown for tetrahedral angles. The experimental results summarized in Table IV show that the change from low pH form to the high pH form results simultaneously in an increase in V_{zz} with a decrease in η. If one assumes as above that the field gradient from each single bond is close to that of a 5p orbital, then because the low pH form of about 100 MHz is six times lower than the value from a 5p orbital V_{zz} correspond to ⅓ in the unit of Figure 7. This means that the experimental results are only in agreement with the point charge model for tetrahedral angles if the low pH form has $S_3 \simeq S_4 \simeq 1$ and the high pH form has either S_3 or S_4 effectively different from 1. We get the experimentally determined V_{zz} and η for the two forms when $S_3 = 0.9$ and $S_4 = 1.2$ for the low pH form and $S_3 = 0.9$ and $S_4 = 1.6$ for the high pH form, using for $S = 1$ a field gradient of 70% of that of a 5p orbital per ligand corresponding to 2.1 pseudo charge per ligand in 2 Å distance. Small changes in the angles of the order of 10° away from pure tetrahedral geometry mainly affect the η values when S_3 and S_4 are close to one, whereas V_{zz} and η in other regions are only to a minor degree affected.

The observed frequency distribution, here assumed to be of a Gaussian form, must mean that several close lying configurations for the metal complex are occupied. The distribution might have been produced by vibrations of one or several of the ligands.

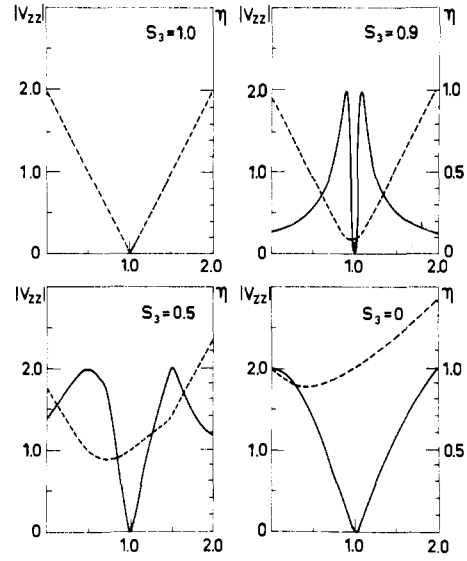

FIGURE 7: The numeric field gradient magnitude $|V_{zz}|$ (- - -) and the asymmetry parameter η (—) as function of the strength of the field gradient of the fourth ligand (S_4) for four selected values of the field gradient strength of the third ligand (S_3). It is assumed that the residual has $S_1 = S_2 = 1$. The r_i^{-3} parameter in eq 14 has been set to 1 which means that a single point charge ligand gives a field gradient $|V_{zz}| = 2$.

A number of different proposals for the catalytical mechanism have been put forward in the current literature on carbonic anhydrase.

It has been suggested (Koenig and Brown, 1972; Lanir et al., 1975) that a water molecule is directly linked to the metal ion at high pH values, while no water molecule is bound at low pH values. Under the assumptions stated above for the angles this suggestion implies that a simultaneous decrease in V_{zz} and increase in η would be expected for the change from low pH to high pH. This is the opposite of the observed change in the PAC spectra described here. In order to achieve agreement between the PAC data analyzed within the point charge model and this proposal for the mechanism, rather large changes in the angles in the direction of the trigonal pyramid (Table I) must be assumed.

In a recent paper (Pesando, 1975) the group critical for the catalytic activity is proposed to be a histidine side chain (His-119) that binds to the metal as anion at high pH values only. This proposal is mainly based on pulsed magnetic resonance study of the histidines in HCAB and HCAC. These data agree well with the work of Campbell et al. (1974), but the latter gives a different interpretation which supports the water–hydroxide mechanism. The proposal put forward by Pesando implies a low pH form which has two protein ligands and possibly a water ligand to Zn. Such a situation is not consistent with the assumptions (1–3) stated above because that would have produced a much larger field gradient than observed. Furthermore Pesando (1975) reports measurements on the Cd enzyme and fails to see any titration of the resonances assigned to the histidine metal ligands in the pH interval 5.5–10.0. This has been taken as implication that Cd binds to the exchangeable histidine over the entire pH region thus staying in the high pH form, which then must be assumed to be inactive. This is in contradiction with both our activity measurements and our observed transitions in the PAC spectra with pH.

Many investigators, however, believe that the catalytical mechanism involves a metal bound water molecule that dissociates a proton at high pH values (Lindskog and Coleman, 1973, Prince and Wolley, 1973). In the point charge model this would require the addition of one unit charge extra on the fourth ligand at high pH, or if one assumes 2.1 pseudo charge on each ligand as used above, an increase of 0.5 unit in S_4 which agrees well with the above estimated change in $S_4 \simeq 0.4$.

In order to reach a more definite conclusion about the nature of the group important for activity, measurement on the C form as well as on the crystallized B form is being prepared. Experiments on the C form should be able to determine whether the difference between the two forms can be explained solely by a decrease of the strength of S_3, i.e., the main difference is that the bond length of N_{94} is larger in the C form than in the B form (K. K. Kannan and I. Vaara, private communication) and experiments on the crystallized enzyme should be able to verify or repudiate the above assumption that the enzyme in solution is close to the crystallized enzyme. Furthermore, experiments performed on a single crystal of the enzyme will be able to give five parameters as mentioned in the theoretical section whereby it should be possible to check the validity of using both the point charge model and tetrahedral angles for the low and high pH form of the enzyme.

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