CARBONIC ANHYDRASE

By
Randara Pulukkody

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Function of Carbonic Anhydrase

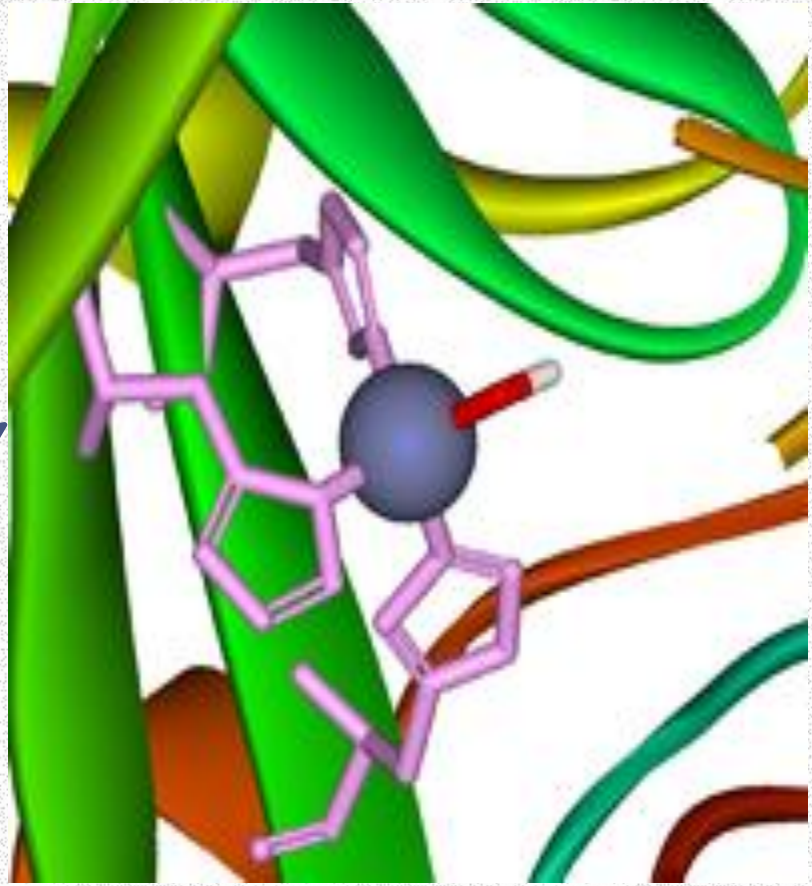
- The **carbonic anhydrases** (CA) form a family of enzymes that catalyze:

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$$

- The transport of CO_2 around the respiratory system is vital, however the solubility of CO_2 in water at physiological conditions is very small
- Carbonic anhydrase enhances the solubility of CO_2 by catalyzing its conversion to the more soluble HCO_3^- ion
- In mammals, the HCO_3^- ion can then be transported to the lungs by the blood stream where it is converted back to CO_2 and exhaled

The Enzyme..

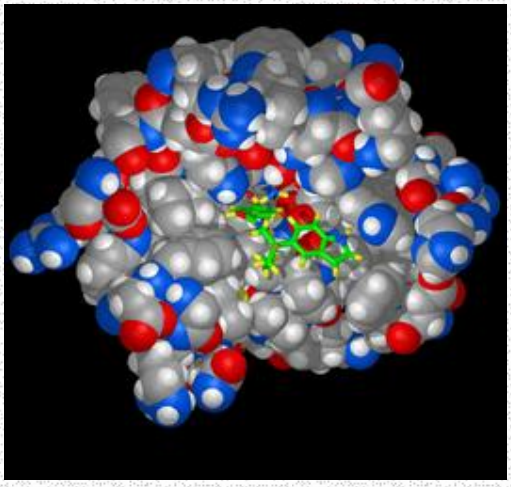
Ribbon diagram of human carbonic anhydrase II. Active site zinc ion visible at center



Close-up of active site of human carbonic anhydrase II, showing three histidine residues (in pink) and a hydroxide group (red and white) coordinating the zinc ion (purple).

http://en.wikipedia.org/wiki/Carbonic_anhydrase

Active Site Architecture..



- Active site : a large, cone-shaped cavity
  15 Å wide & 15 Å deep
- Zinc (II) ion : ligated by 3 Histidines
  near the bottom of the cavity
- 4^{th} ligand : OH^-
- Hydrophilic component : order several water molecules for proton transfer
- Hydrophobic patch: pre-organizes the CO_2 substrate
  squeezes the HCO_3^- product from the active site

Lindskog, S. (1997). "Structure and mechanism of carbonic anhydrase." Pharmacology & Therapeutics **74**(1): 1-20.

Discovery of CA

"Uncatalyzed rate of HCO_3^- dehydration is too low to support CO_2 excretion during the time blood spent at the gas exchange surface"

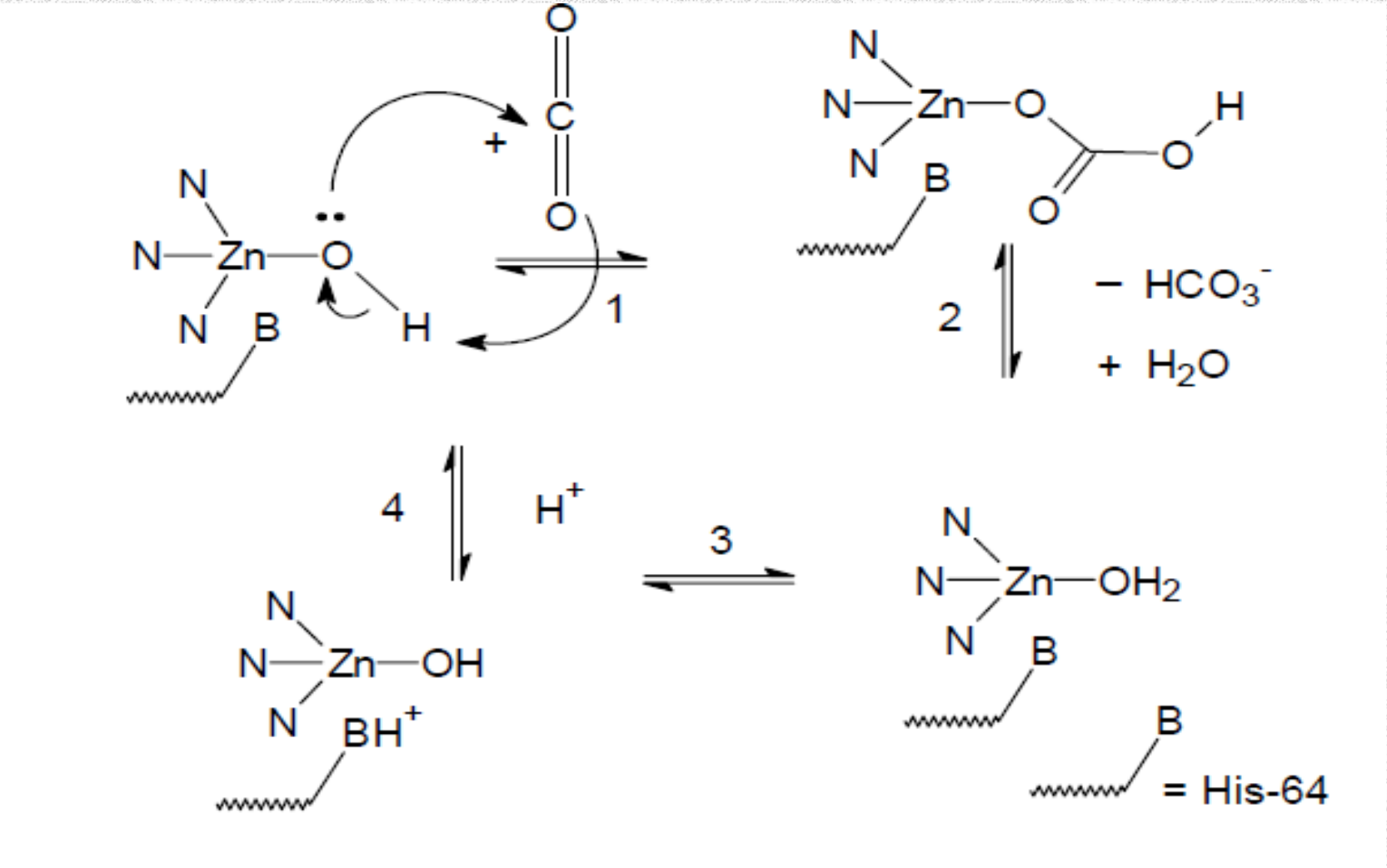
*Frances J W Roughton

*Norman U Meldrum

- CA in broad interest because:
  - One of the fastest enzymes known;
    turnover number(k_{cat}) $>1\times10^6\ s^{-1}$
  - Fundamental to a wide array of physiological processes;
    may be among the earliest enzymes to appear

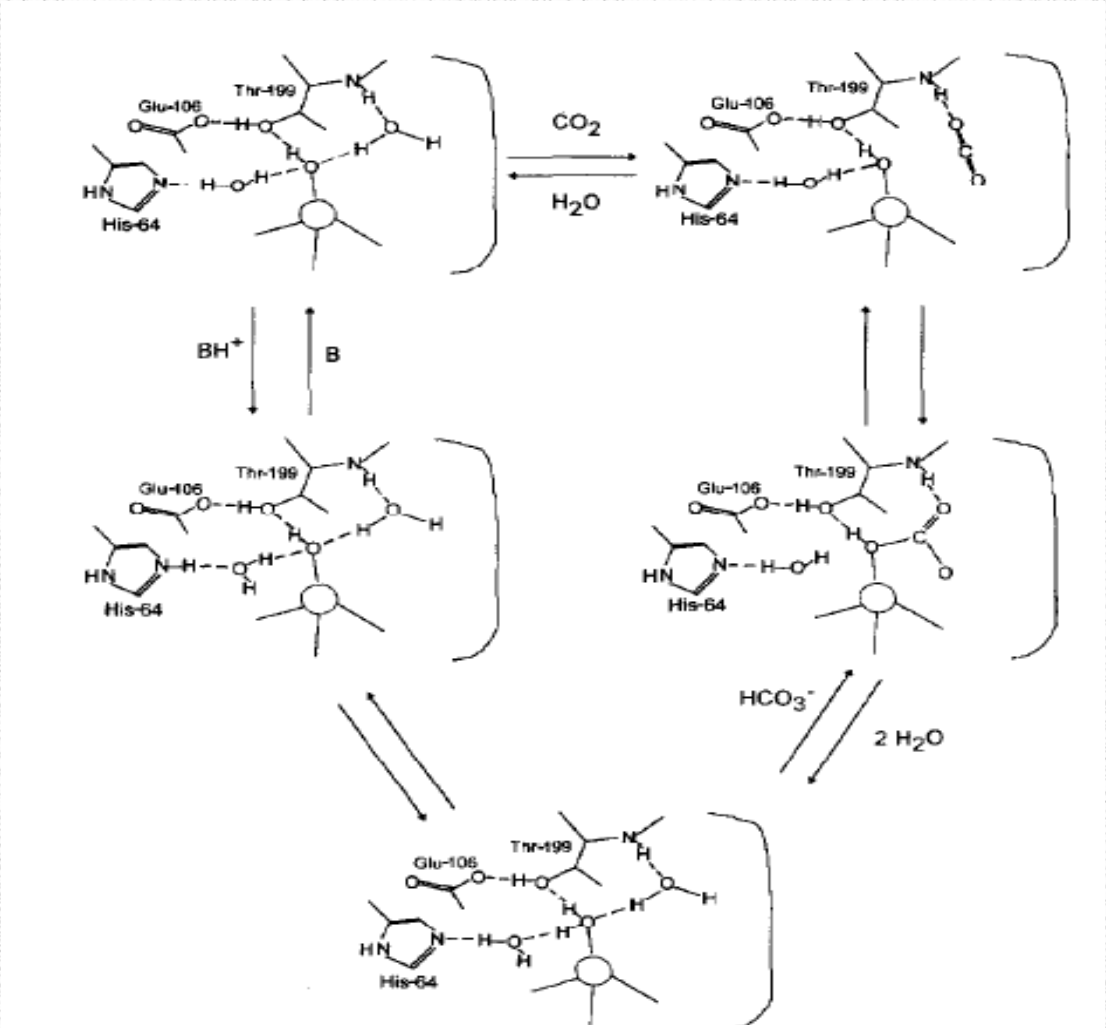
*The Carbonic anhydrases: New horizons (Text Book)

Simple Mechanism



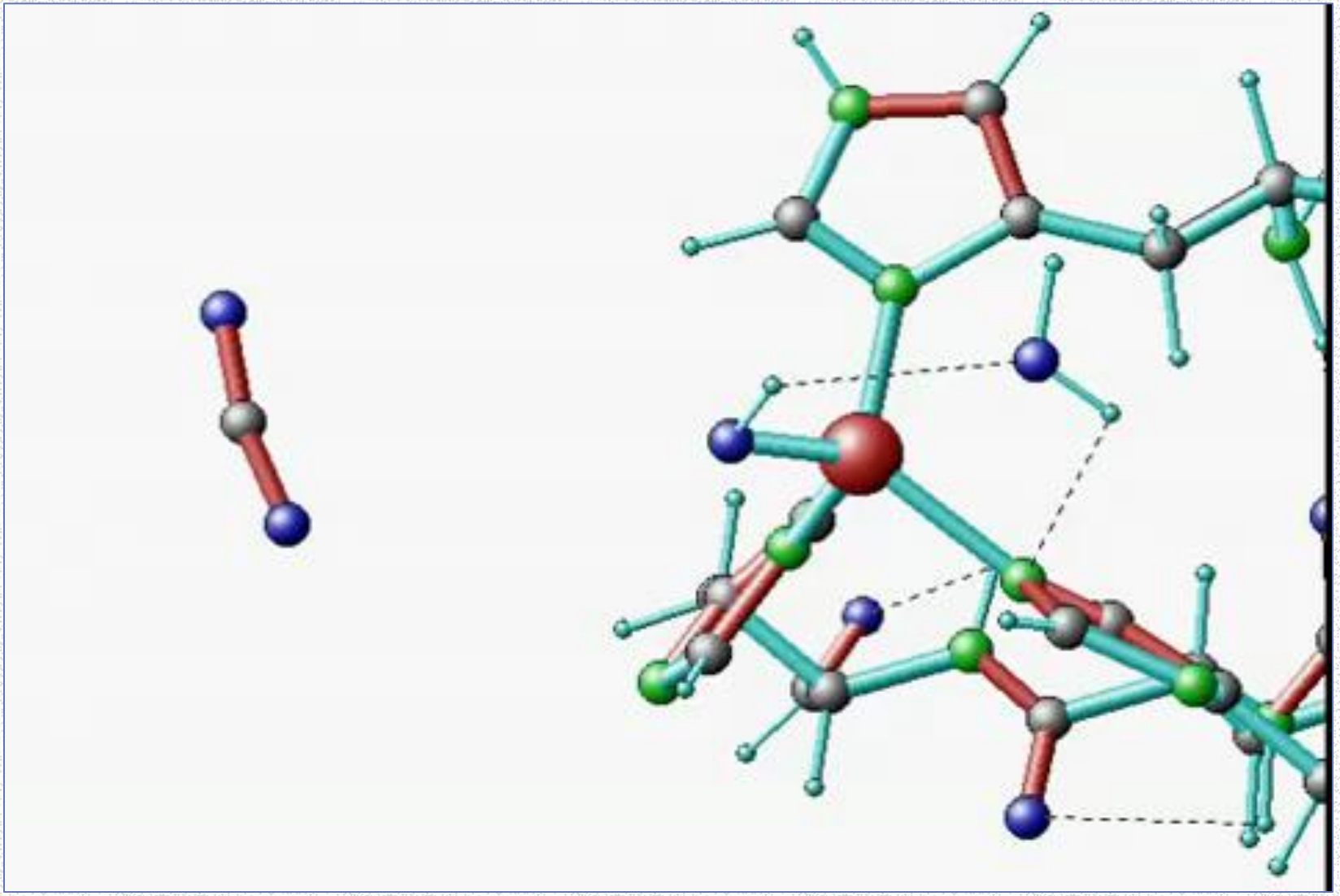
http://www.waltry.f2s.com/lectures/bioinorg-lec7.pdf

The Full Mechanism



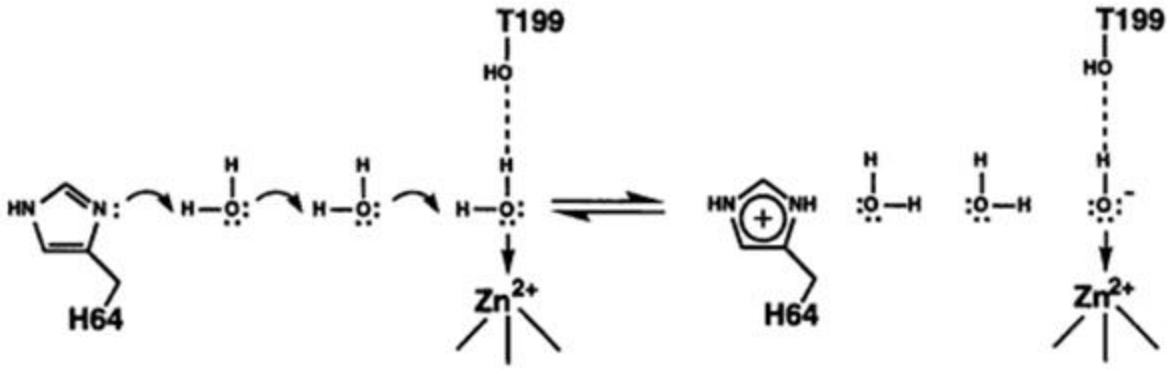
Scheme of the catalytic mechanism of human CA

Lindskog, S. (1997). "Structure and mechanism of carbonic anhydrase." Pharmacology & Therapeutics **74**(1): 1-20.



The Proton Shuttle

- His 64 identified as proton shuttle
- Hydrogen bonded through two bridging solvent molecules to the zinc bound hydroxyl group
- Proton transfer occurs across the bridging solvent network : proton “translocation”



Mechanism of proton transfer between zinc-bound water and His-64. Once protonated, His-64 transfers a proton to a buffer molecule in bulk solvent. If the solvent-mediated “proton wire” is perturbed, then the rate of proton transfer will be substantially diminished.

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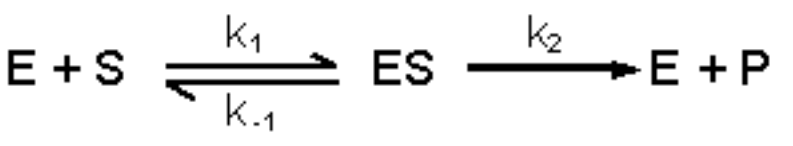
Enzyme Kinetics: Michaelis-Menten Equation



- Using Steady State Approximation;

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

where;

$$K_m = \frac{k_2 + k_{-1}}{k_1}$$

Michaelis-Menten Equation

V_0 – Initial velocity

V_{max} – Maximum velocity

E – [Enzyme]

k_M – Michaelis Constant

S – [Substrate]

P – [Product]

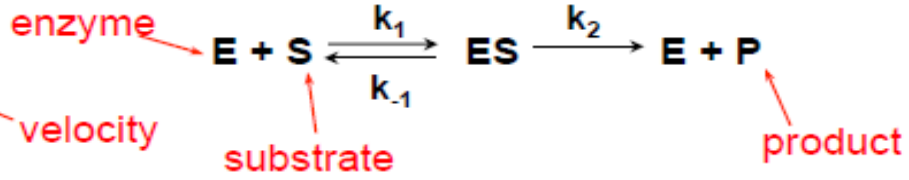
ES – [Enzyme-substrate complex]

k_{cat} – turnover number for the enzyme

k_{cat}/k_M – specificity constant

ENZYME CATALYSIS

Simple Michaelis-Menten Kinetics:



$$R = \frac{d(P)}{dt} = k_2(ES) = v$$

velocity

Steady-state approx. on ES:

$$\frac{d(ES)}{dt} = 0 = k_1(E)(S) - k_{-1}(ES) - k_2(ES)$$

$$E_o = (E) + (ES) \quad \text{or} \quad (E) = E_o - (ES)$$

E_o is the total amount of enzyme present

$$k_1E_o(S) - k_1(ES)(S) - k_{-1}(ES) - k_2(ES) = 0$$

$$(ES)_{ss} = \frac{k_1E_o(S)}{k_{-1} + k_2 + k_1(S)}$$

$$v = \frac{k_1k_2E_o(S)}{k_{-1} + k_2 + k_1(S)} = \frac{k_2E_o(S)}{\frac{k_{-1} + k_2}{k_1} + S} = \frac{k_2E_o(S)}{K_m + (S)}$$

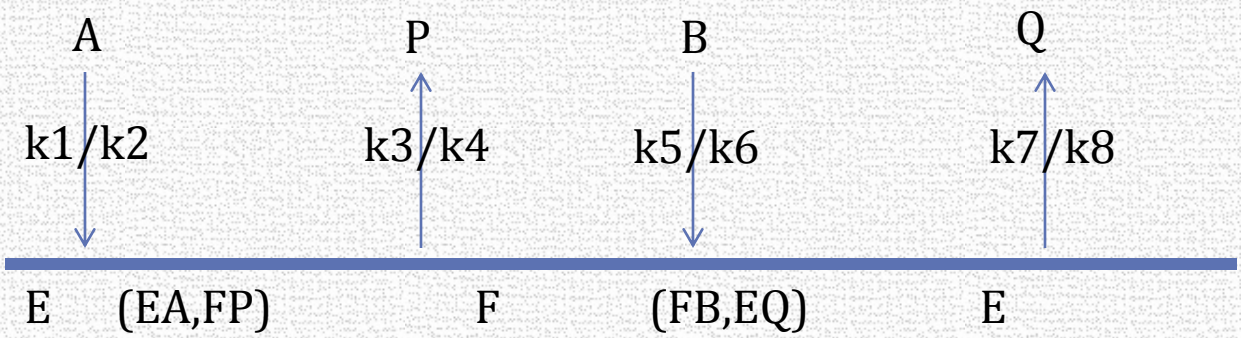
This is the Michaelis-Menten Equation.

Michaelis constant

http://course.ucsf.edu/pc111/PowerptNotes/8.enzyme_catalysis.pdf

Ping-Pong Mechanism

- A type of multisubstrate mechanism
- A product is released before all of the substrates are bound.
- Eg: Ping – Pong Bi Bi Mechanism



- E- unsubstituted enzyme
- F- substituted enzyme

Implications of a Rate-Limiting Protolysis of Water

- Suggested that the rate-limiting step is the protolysis of water rather than the new carbon-oxygen bond formation in HCO_3^-

$$H_2O \rightleftharpoons OH^- + H^+$$

- Mechanism proposed:

$$EZnOH^- + CO_2 \rightleftharpoons EZn(OH^-)CO_2 \rightleftharpoons EZnHCO_3^- \rightleftharpoons EZnH_2O + HCO_3^- \quad (3)$$

$$EZnH_2O \rightleftharpoons EZnOH^- + H^+ \quad (4)$$

- Direct nucleophilic attack of Zn-bound OH^- to CO_2
- PING-PONG type mechanism: Interconversion between CO_2 and HCO_3^- is temporally separated from the release of the proton

Silverman, D. N. and S. Lindskog (1988). "The Catalytic Mechanism of Carbonic-Anhydrase - Implications of a Rate-Limiting Protolysis of Water." Accounts of Chemical Research **21**(1): 30-36.

release of H^+ from the active site presents a problem

$$EZnOH^- + CO_2 \rightleftharpoons EZn(OH^-)CO_2 \rightleftharpoons EZnHCO_3^- \rightleftharpoons EZnH_2O + HCO_3^- \quad (3)$$

$$EZnH_2O \rightleftharpoons EZnOH^- + H^+ \quad (4)$$

- Maximal rate constant for the transfer of a proton from a catalytic group of pKa = **7** to bulk water is about $\mathbf{10^3}$ s^{-1} : *1000 slower than the maximal turnover number

- $[OH^-]$ which is a good proton acceptor, is too small at physiological pH to provide an explanation of the large catalytic turnover.

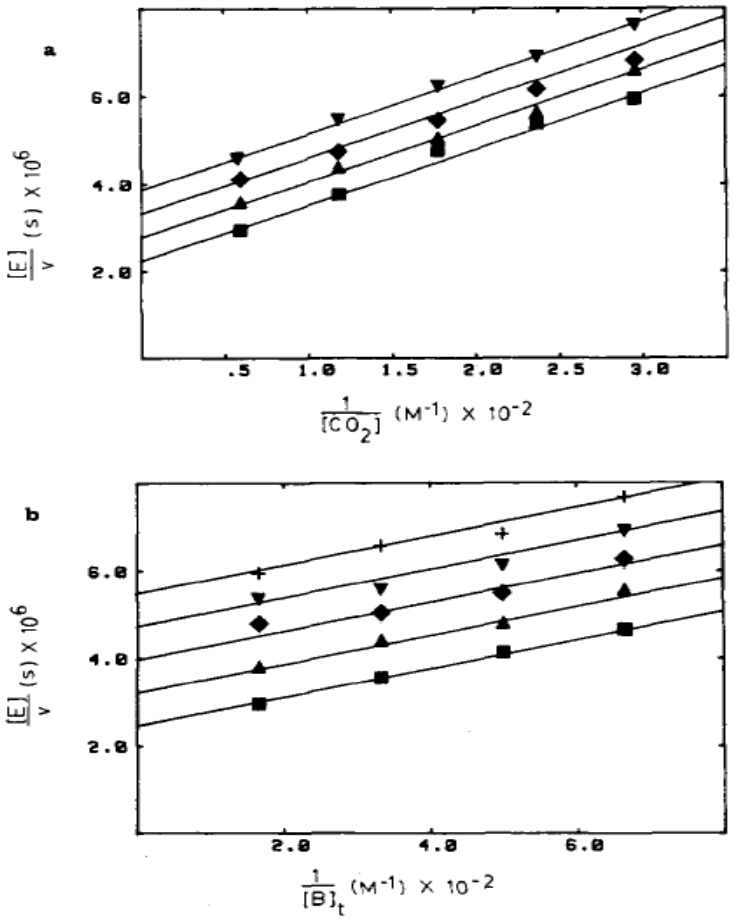
- Alberty and Eigen and Hammes :

  buffers in solution are involved

  - much better proton acceptors than H_20 and
  - much more concentrated at physiological pH than OH^-

Silverman, D. N. and S. Lindskog (1988). "The Catalytic Mechanism of Carbonic-Anhydrase - Implications of a Rate-Limiting Protolysis of Water." Accounts of Chemical Research **21**(1): 30-36.

Verification of the involvement of Buffers



Two double-reciprocal plots showing the parallel patterns characteristic of Ping-Pong mechanisms for both CO_2 (top) and buffer (bottom) as substrates. The initial velocity of catalyzed hydration of CO_2 was measured by stopped flow using a changing pH indicator method; $[B]_t$ is the total concentration of buffer. Human red cell carbonic anhydrase II was present at 69 nM, and ionic strength was maintained at 0.2 M with Na_2SO_4. Temperature was 25 °C and pH 8.5. (top) [1,2-dimethylimidazole] = 6.0 mM (■), 3.0 mM (▲), 2.0 mM (◆), 1.5 mM (▼). (bottom) Replot of the same data with B = 1,2-dimethylimidazole, $[CO_2]$ = 17 mM (■), 8.5 mM (▲), 5.6 mM (◆), 4.2 mM (▼), 3.4 mM (+). Reproduced from ref 13.

- As the buffer concentration was decreased:
  - Decrease in the initial velocity
- Buffers in solution participate as proton-transfer agents

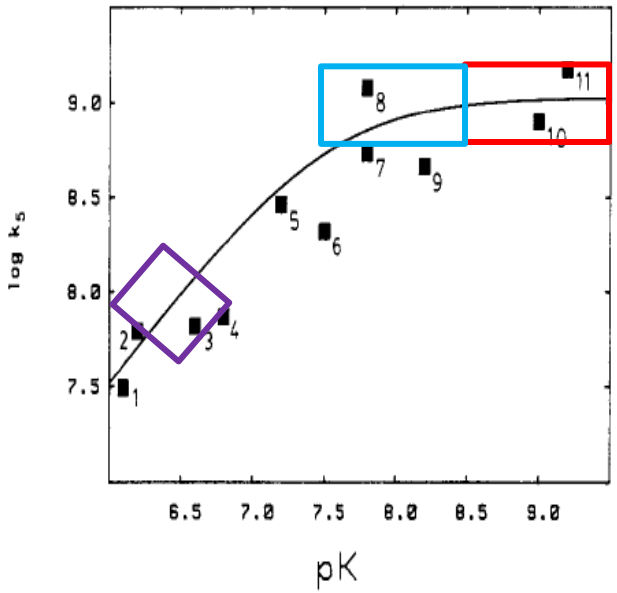
$$EZnH_2O + \text{buffer} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EZnOH^- + \text{buffer}H^+ \quad (5)$$

- Initial velocity patterns consistent with Ping-Pong mechanism of eq3 and eq5
- Verify the initial hypothesis:

  Intermolecular H^+ transfer occurs in a step separate from the inter-conversion of $C0_2$ and HCO_3^-

Further Evidence

- 13C **NMR** measurements of rates of the inter-conversion between $C0_2$ and $HC0_3^-$ at equilibrium showed no buffer effect



The variation of the logarithm of k_5 of eq 5 showing a dependence on the pK_a of the external buffers as proton acceptors very similar to the plots of proton transfer between small molecules described by Eigen.[25] The external buffers are (1) Mes, (2) 3,5-lutidine, (3) 3,4-lutidine, (4) 2,4-lutidine, (5) 1-methylimidazole, (6) Hepes, (7) triethanolamine, (8) 4-methylimidazole, (9) 1,2-dimethylimidazole, (10) Ted, and (11) Ches. The curve drawn through the points was calculated for $k_5 = 1.1 \times 10^9$ M^{-1} s^{-1} and a pK_a for the donor group on the enzyme of 7.6. Re-

- Interaction between enzyme and buffer has little structural specificity
- When pK_a of the buffer > pK_a of the catalytic group;
  - k_5 independent of the pK_a value **of** the buffer
  - k_5 =**10^9** M^{-1} s^{-1} - a diffusion-controlled process
- When pK_a donor = pK_a acceptor;
  - Transition
- When pK_a of the buffer < pK_a of enzyme as donor;
  - region of the plot of slope equal to unity
- Indicates a pK_a of the donor group on the enzyme = **7.6 ± 0.6**

Silverman, D. N. and S. Lindskog (1988). "The Catalytic Mechanism of Carbonic-Anhydrase - Implications of a Rate-Limiting Protolysis of Water." Accounts of Chemical Research **21**(1): 30-36.

Intramolecular Proton Transfer

- Hypothesis by Steiner *et al.*:

> H^+ donor of $pK_a \approx 7$ previously described is not the zinc-bound water at all but another residue closer to the surface of the enzyme

Testing..

- Sufficiently high [buffer] used to ensure that intermolecular proton transfer is not rate-limiting
- Compared the Michaelis - Menten parameters for carbonic anhydrase II in H_2O and D_2O

Results..

- Isotope effect of 3.8 in k_{cat} for hydration
- Isotope effect of 1 in the ratio k_{cat}/k_M

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Interpretation

$$EZnOH^- + CO_2 \rightleftharpoons EZn(OH^-)CO_2 \rightleftharpoons EZnHCO_3^- \rightleftharpoons EZnH_2O + HCO_3^- \quad (3)$$

$$EZnH_2O + buffer \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EZnOH^- + bufferH^+ \quad (5)$$

k_{cat}/k_M

- k_{cat}/k_M: contains rate constants for steps from the initial encounter of substrate with enzyme through the first irreversible step ⟶ **Departure of product HCO_3^- from the enzyme**

- Thus, k_{cat}/k_M contains rate constants for eq3 only, **not** eq5
- Isotope effect of 1 in the ratio k_{cat}/k_M

Steps in eq3 do not involve a change in bonding to H in a rate-contributing step

Conclusions

Inter-conversion of CO_2 and HCO_3^- occurs by direct nucleophilic attack of zinc-bound hydroxide on CO_2 without rate-contributing proton transfer

No general base mechanism in which zinc-bound hydroxide abstracts a proton from an adjacent water

k_{cat}

Eliminated rate-contributing intermolecular proton transfer: large [buffer] used

Isotope effect of 3.8 in k_{cat} for hydration:

Large enough to indicate a primary intramolecular proton transfer in the catalysis

$$EZnOH^- + CO_2 \rightleftharpoons EZn(OH^-)CO_2 \rightleftharpoons EZnHCO_3^- \rightleftharpoons EZnH_2O + HCO_3^- \quad (3)$$

$$EZnH_2O + \text{buffer} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EZnOH^- + \text{buffer}H^+ \quad (5)$$

k_{cat} contains rate constants for the entire catalysis

Overall isotope effect = 1

Intramolecular proton transfer involved

suggested that proton transfer was the protolysis of zinc-bound water by transfer of a proton to a nearby residue of the enzyme with a similar pK, value near 7

Hints on the proton shuttle

- X-ray diffraction structure of HCA II suggested His-64 as the foremost candidate for this nearby residue
- His-64 :
  - pK_a = 7.1
  - Situated 6 Å from the Zn
- The proton is transferred from His-64 to buffer in solution completing the catalytic cycle

His-64 is a “proton shuttle”

- Yet, transfer of H^+ between the metal site and His-64 seems to be the "most difficult" step in the catalysis.

Bridging Water Molecules

- Thorough investigation of the solvent hydrogen isotope effect on k_{cat} for hydration showed an exponential dependence on the atom fraction of deuterium in solvent water

- This result strongly suggests proton transfer through intervening water bridges

- Later confirmed to be correct by detailed interpretation of the refined crystal structure of carbonic anhydrase II