<u>Lecture #4:</u> Graphical Analysis of Kinetic Data, pH, Temp Dependence and Sigmoidal Kinetics

(1) Graphical Analysis

(a) Lineweaver-Burk Analysis

(b) Hanes-Woolf Analysis

(c) Eadie-Hofstee Analysis

(d) Direct Linear Plot (Eisenthal/Cornish-Bowden Plot)

(e) Nonlinear Curve Fitting

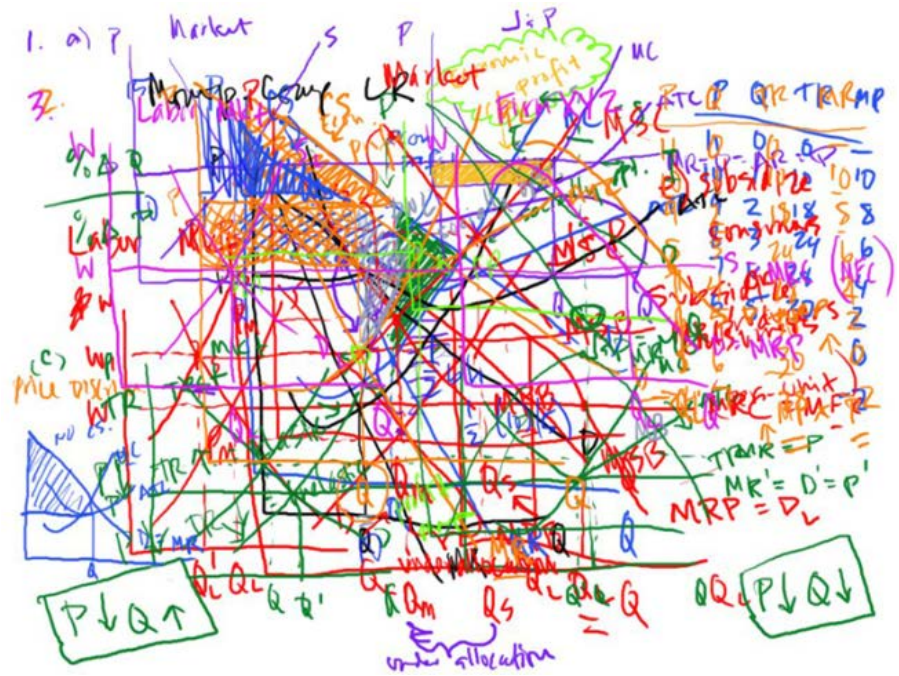
(2) pH-dependence of Michaelis-Menten Enzymes

-pK_a shifts of active site residues

(3) Temperature-dependence of Enzyme Reactions

- Arrhenius plots
- Derivation of Ionizable Equation

(4) Allosteric enzymes



Lecture # 4: **Graphical Analysis of Kinetic Data, pH, Temp Dependence and Sigmoidal Kinetics**

1. Graphical Analysis

- aim is to calculate K_M and V_{max} using graphs of the terms v_o and $[S]_o$

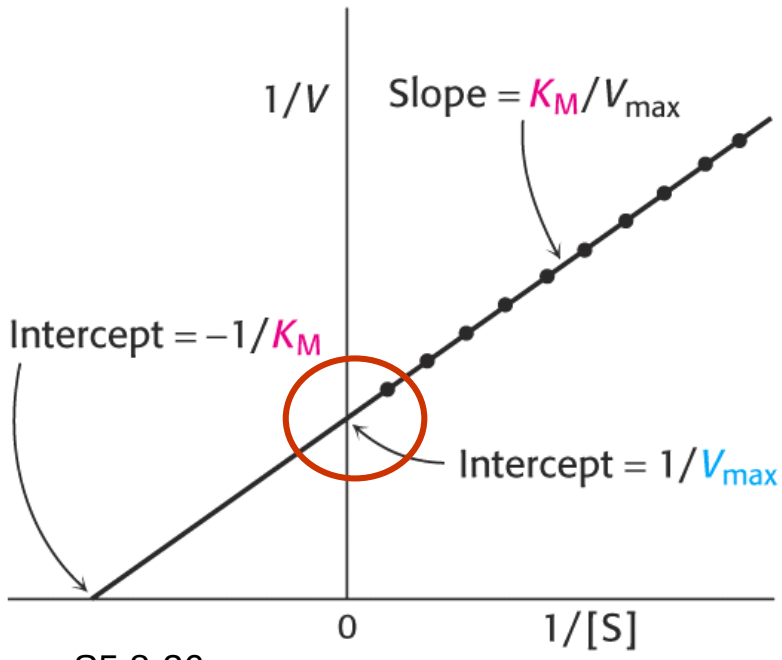
(a) Lineweaver-Burk Analysis (1934)

Recall: $$v_o = \frac{V_{max}[S]}{K_M + [S]}$$

Invert: $$\frac{1}{v_o} = \frac{K_M + [S]}{V_{max}[S]}$$

Separate terms: $$\frac{1}{v_o} = \frac{K_M}{V_{max}[S]} + \frac{[S]}{V_{max}[S]}$$

Plot $1/v_o$ *vs* $1/[S]$:



S5:8-36

Lineweaver-Burk plot

$$\underset{Y}{\frac{1}{v_o}} = \underset{m}{\left(\frac{K_M}{V_{max}}\right)}\underset{x}{\frac{1}{[S]}} + \underset{b}{\frac{1}{V_{max}}}$$

Y = mx + b

- most commonly used plot
- departures from linearity less obvious
- undue weighting due to high [S] clustering near origin

(b) Hanes—Woolf Analysis

Recall:

$$\frac{1}{v_o} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

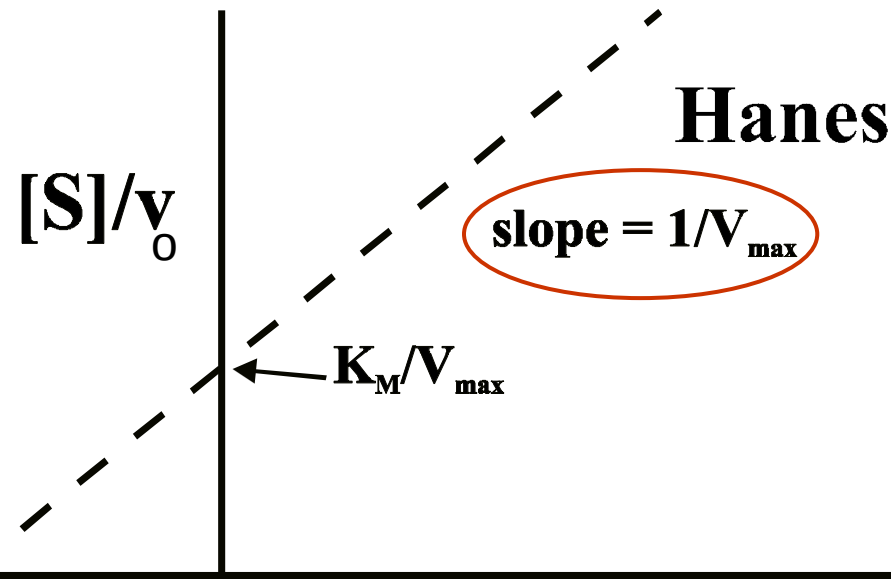
L-B transformation

Multiply by [S]:

$$\frac{[S]}{v_o} = \frac{K_M}{V_{max}} + [S]\left(\frac{1}{V_{max}}\right)$$

Y = b + Xm

Plot $[S]/v_o$ *vs* [S]:



- good plot for V_{max} determination (slope)
- avoids problem of velocity (independent) variable (x axis) influencing the dependent variable (y axis) as per Eadie-Hofstee
- not as good for K_M determination (intercept)

(c) Eadie-Hofstee Analysis

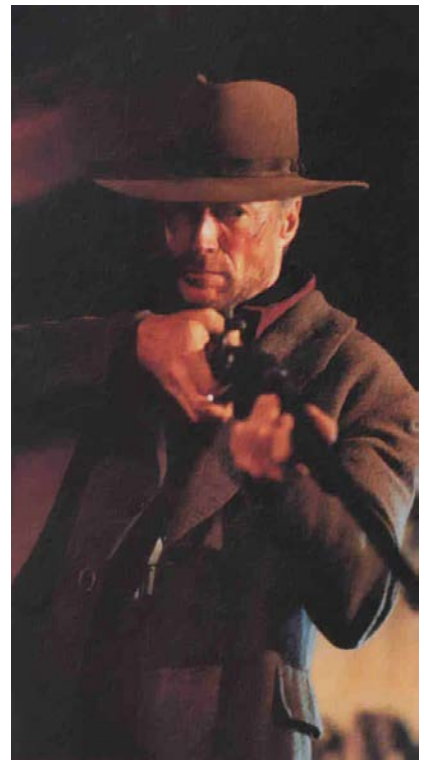
Recall: $$\frac{1}{v_o} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$ L-B transformation

Multiply by $v_o V_{max}$: $$\frac{v_o V_{max}}{v_o} = \frac{K_M\, v_o V_{max}}{V_{max}[S]} + \frac{v_o V_{max}}{V_{max}}$$

Reduces to: $$V_{max} = \frac{v_o K_M}{[S]} + v_o$$

Solve for v_o: $$v_o = V_{max} - K_M \left(\frac{v_o}{[S]}\right)$$ (Y = v_o, X = $\frac{v_o}{[S]}$)

Plot v_o vs $v_o/[S]$:



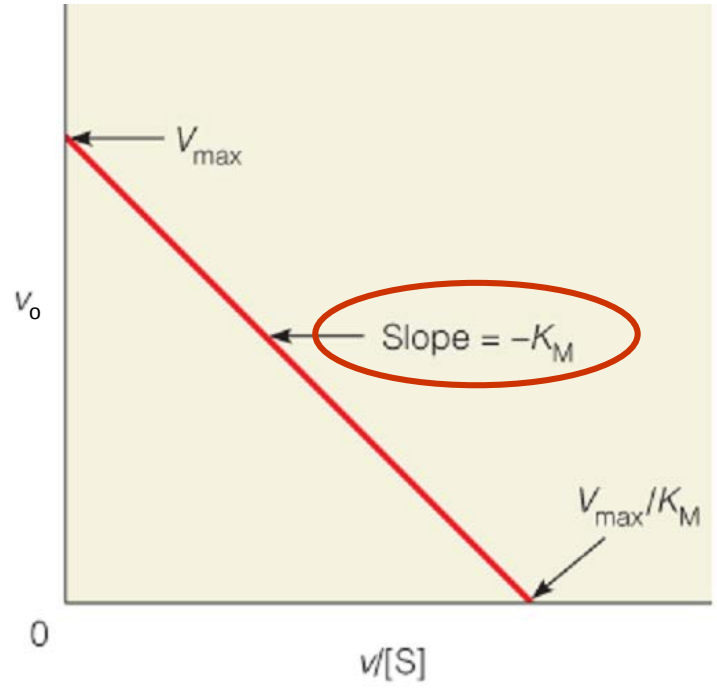


FIGURE 11.26 Biochemistry Matthews 2013

An Eadie–Hofstee plot. Graphing v versus v/[S], we obtain V_{max} at (v/[S]) = 0 and K_M from the slope of the line.

-good for K_M determination (slope)

-errors in velocity (v_o) will be correlated in the plot since v_o is in both the independent and dependent variables

(d) Direct Linear Plot (Eisenthal/Cornish-Bowden Plot)

•Eisenthal and Cornish-Bowden (1974) suggested a different approach

Recall: $$\frac{1}{v_o} = \frac{K_M + [S]}{V_{max}[S]}$$ Rearrange

Bring V_{max} over v_o: $$\frac{V_{max}}{v_o} = \frac{K_M + [S]}{[S]}$$

Reduces to: $$\frac{V_{max}}{v_o} = \frac{K_M}{[S]} + 1$$

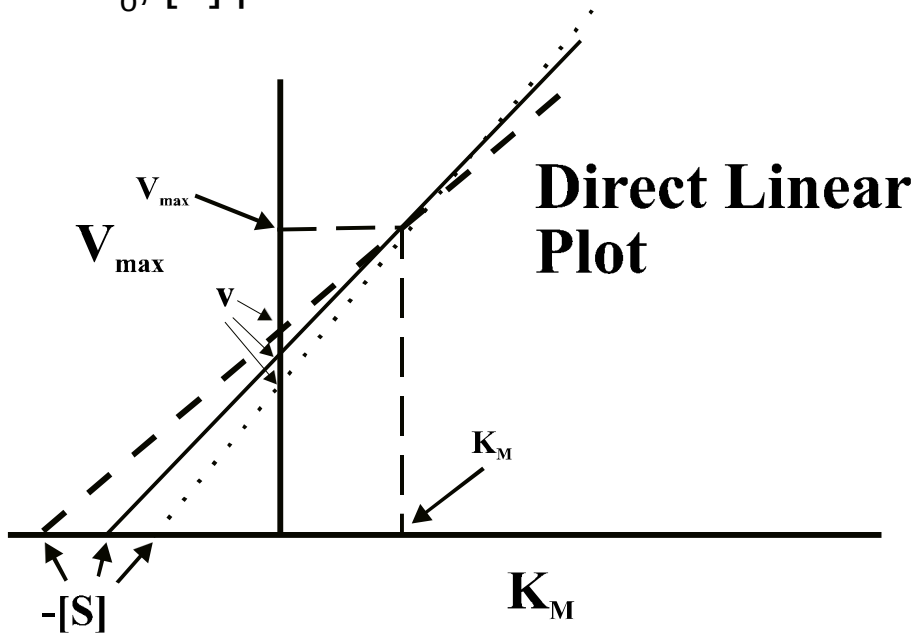
•a plot of V_{max} against K_M (at constant v_o and [S]) is linear

•When $K_M = 0$ then $V_{max} = v_o$

•When $V_{max} = 0$ then $K_M = -[S]$

•Each v_o, [S] pair used to generate a line by marking v_o on the V_{max} axis and –[S] on the K_M axis (0, vo) and (-[S],0)

•Lines for all v_o, [S] pairs should be linear and must pass through the true values of K_M and V_{max}

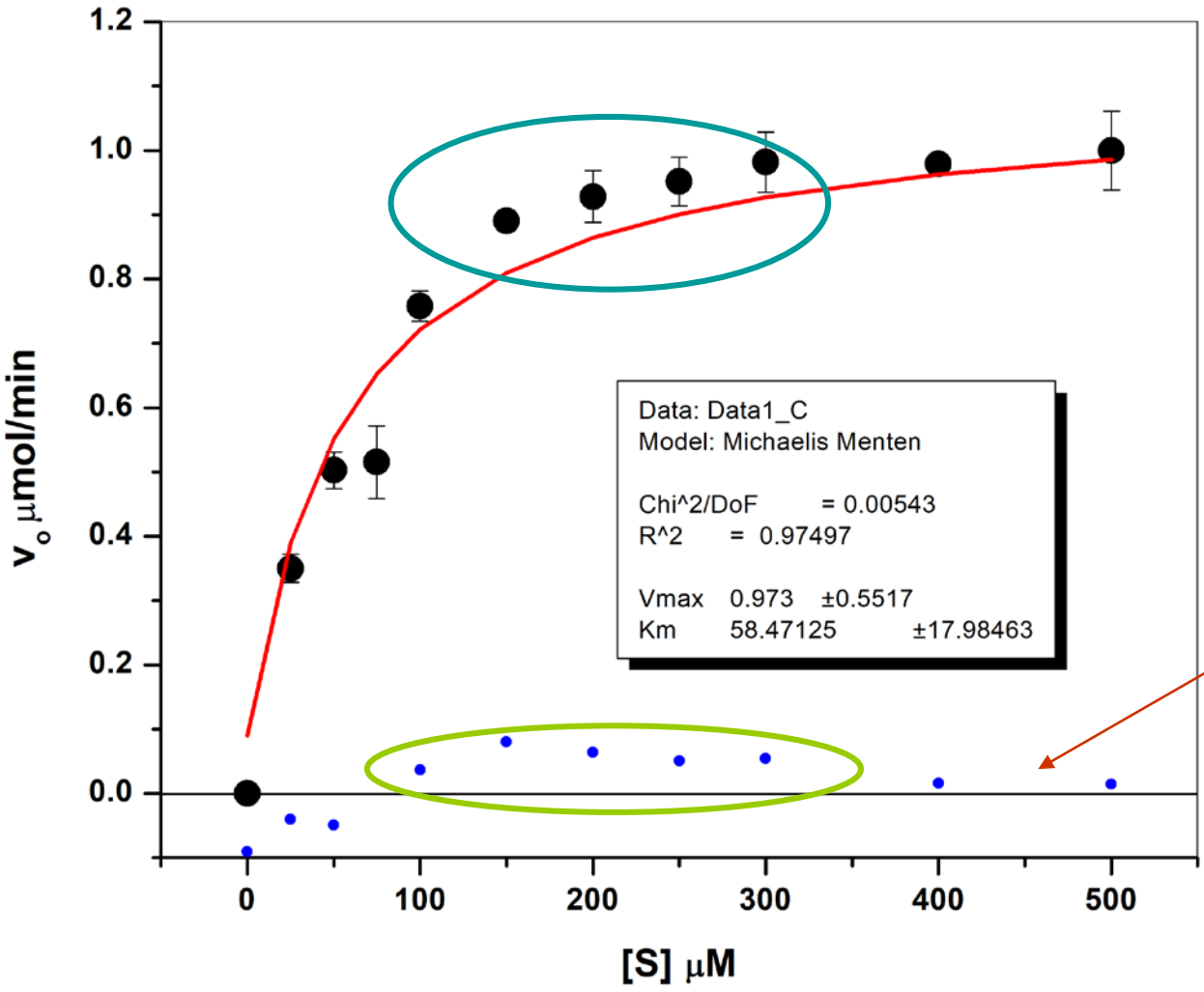


-can plot the v_o, [S] pairs during the acquisition of kinetic data

(e) Nonlinear Curve fitting

•Michaelis-Menten data can be fitted to the M-M equation

$$v_o = \frac{V_{max}[S]}{K_M + [S]}$$



- Advantage: fast and no calculations/transformation required
- Disadvantage: goodness of fit can be difficult to determine accurately; difficult to spot areas of inadequate fit

2. pH-dependence of Michaelis-Menten Enzymes

•initial rates for many enzymatic reactions exhibit bell-shaped curves as a function of pH

•curves reflect the ionizations of certain amino acid residues that must be in a specific ionization state for enzyme activity

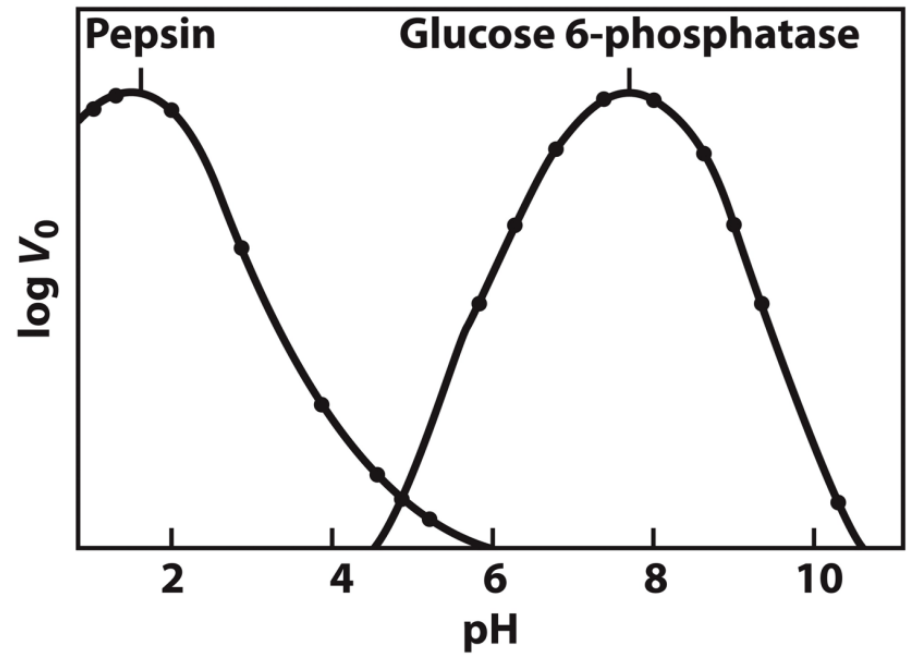


Figure 6-18
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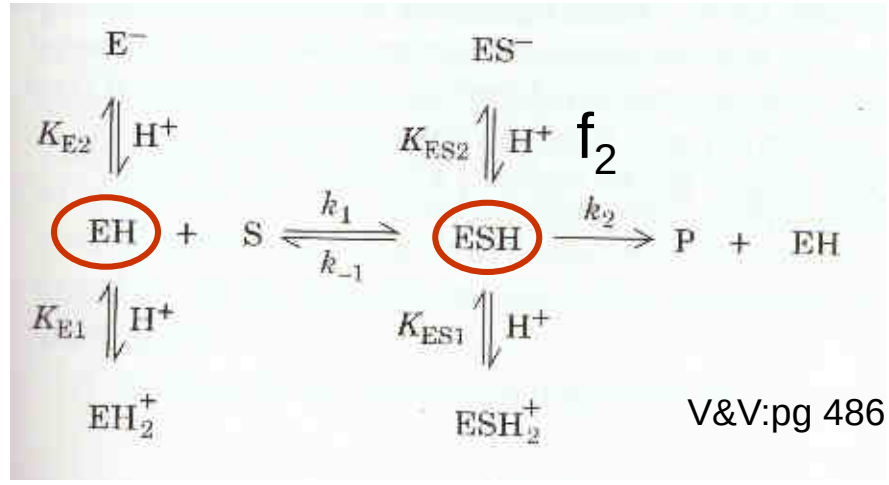
•following model can account for such pH effects



V&V:pg 486

•it is assumed that only EH and ESH are catalytically active
•Michaelis-Menten equation for this model is:

$$v_o = V'_{max}[S]/(K'_M + [S]) \qquad \{pH\text{-}1\}$$

where $V'_{max} = V_{max}/f_2$ and $K'_M = K_M(f_1/f_2)$

and $f_1 = [H^+]/K_{E1} + 1 + K_{E2}/[H^+]$

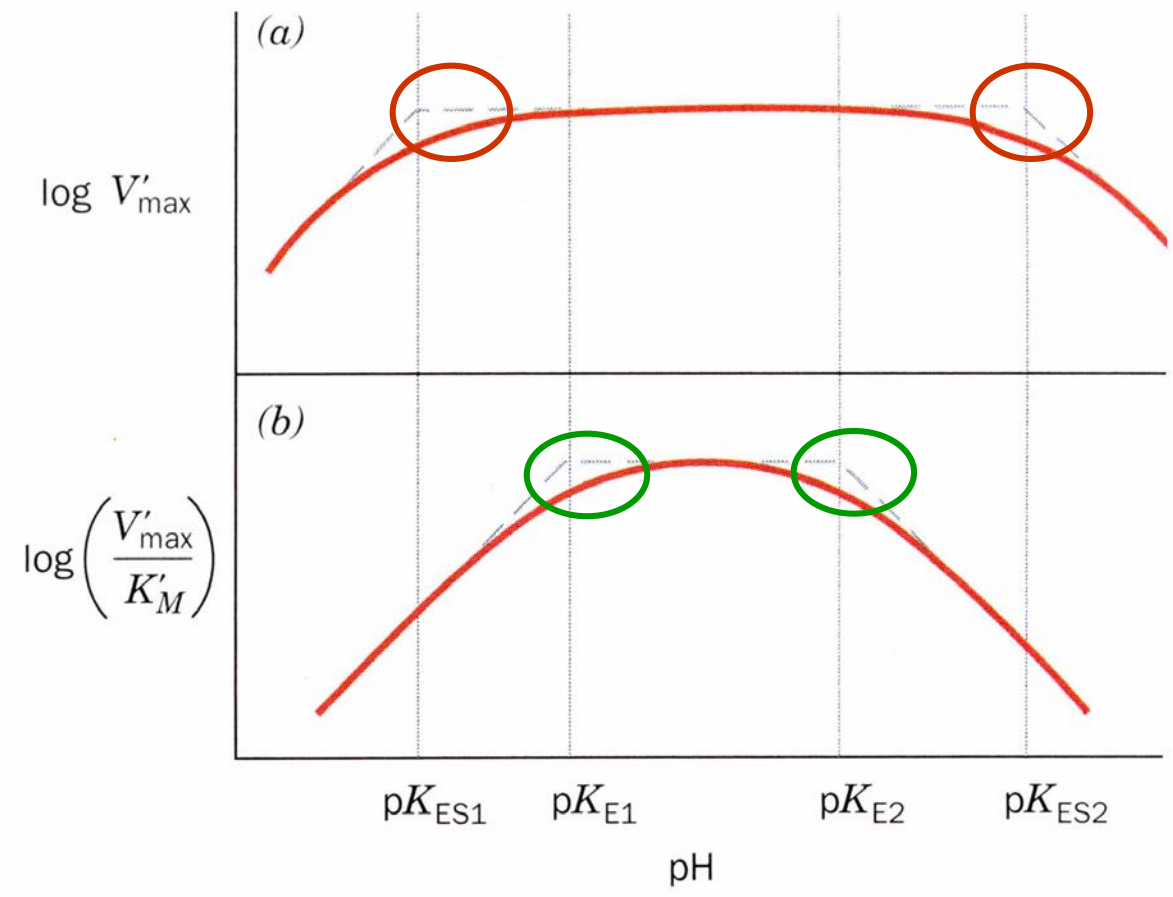
and $f_2 = [H^+]/K_{ES1} + 1 + K_{ES2}/[H^+]$

(derivation in Ch14 V&V,pg 492-493; see Lecture Appendix)

•Here, V_{max} and K_M refer to the active forms of the enzyme, EH and ESH

•At any given pH, equation {pH-1} behaves as a simple Michaelis-Menten equation

- because of the pH-dependence of f_1 and f_2, v_o varies with pH in a bell-shaped manner



V&V:14-16

•the ionization constants of enzymes that obey {pH-1} can be evaluated by the analysis of the curves of log V'_{max} versus pH, which provides values of K_{ES1} and K_{ES2}

•evaluation of a plot of log (V'_{max}/K'_M) vs pH yields K_{E1} and K_{E2}

•experimentally, this entails the determination of the enzyme's Michaelis-Menten parameters at each of a series of different pH values

•measured pK_a's often provide <u>valuable clues</u> as to the identities of the amino acid residues essential for enzymatic activity

•a pK_a of ~4 suggests that an Asp or Glu residue is essential to the enzyme

•a pK_a of~6 suggests the role for an active site His residue

•a pK_a of ~10 suggests the involvement of an active site Lys

Fersht:T5-1

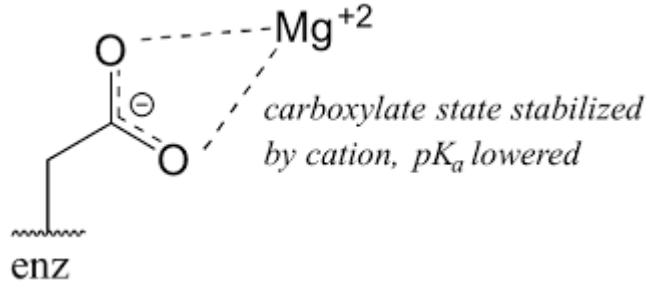
Table 5.1 *pK_a's of ionizing groups*[a]

Group	pK_a: Model compounds (small peptides)	pK_a: Usual range in proteins
Amino acid α-CO_2H	3.6	
Asp (CO_2H)	4.0	2–5.5
Glu (CO_2H)	4.5	
His (imidazole)	6.4	5–8
Amino acid α-NH_2	7.8	~8
Lys (ϵ-NH_2)	10.4	~10
Arg (guanidine)	~12	—
Tyr (OH)	9.7	9–12
Cys (SH)	9.1	8–11
Phosphates	1.3, 6.5	—

[a] Data mainly from C. Tanford, *Adv. Protein Chem.* **17**, 69 (1962); C. Tanford and R. Roxby, *Biochemistry* **11**, 2192 (1972); Z. Shaked, R. P. Szajewski, and G. M. Whitesides, *Biochemistry* **19**, 4156 (1980).

pKa shifts

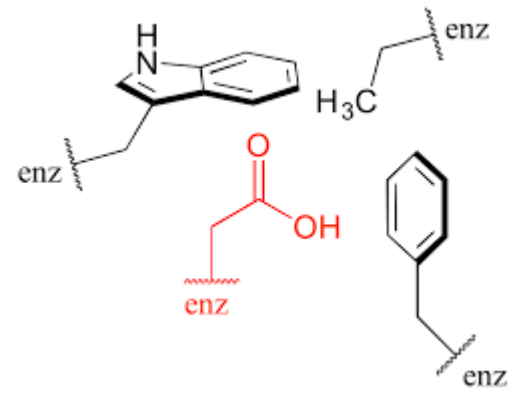
-environmental shifts of pK_a's are a common occurrence in enzyme active sites



e.g., the carboxylate group of an Asp residue forming a complex with Mg^{2+} is stabilized by the nearby positive charge and therefore has a lower pK_a than it would otherwise have

- it means that it is more difficult to protonate
- conversely, a side-chain carboxylate group (e.g., Asp) immersed in a region of low polarity is less acidic than normal because it attracts protons more strongly than if it were in a region of higher polarity

- It means that it is easier to protonate
- It will have a higher pK_a



- the identification of a kinetically characterized pK_a with a specific amino acid residue **must therefore be verified by other types of measurements** such as the use of group-specific reagents to inactivate a putative residue

3. Temperature-Dependence of Enzyme Reactions

1859-1927

Svante Arrhenius
Nobel Prize 1903

Rate Constants and Temperature
(1) Arrhenius Equation

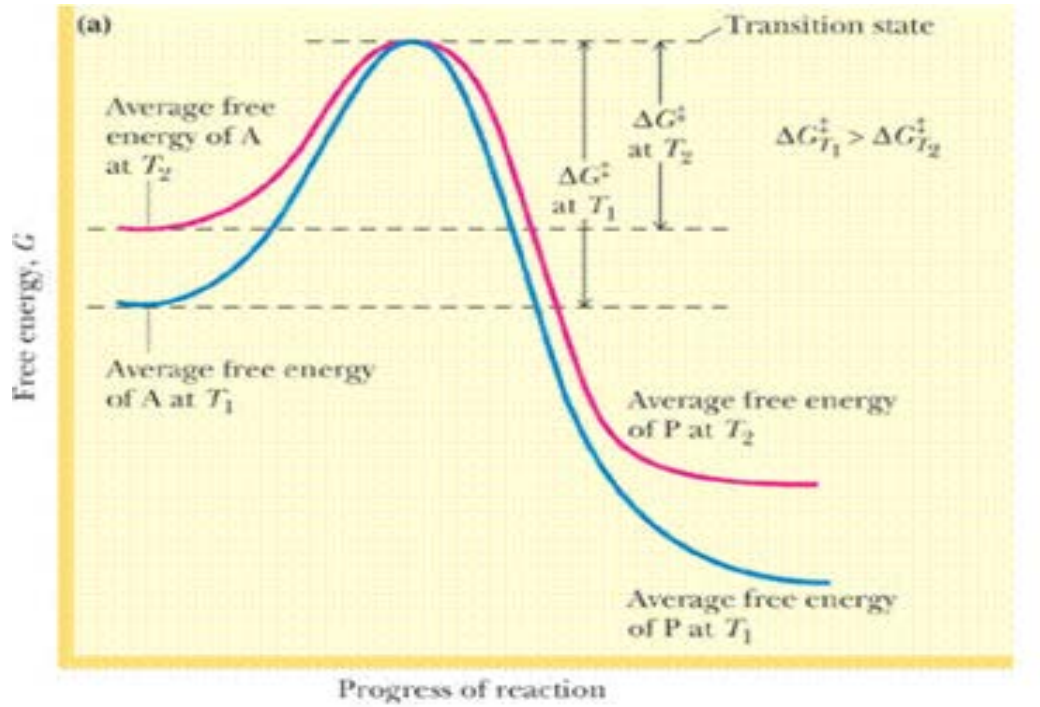
- Svante Arrhenius (1889)

developed an empirical relationship
between rate constants and T

(a) logarithmic relationship between rate constant and 1/T (K)

(b) K_{eq} viewed as ratio of 2 rate constants

$$A + B \underset{k-1}{\overset{k1}{\Leftrightarrow}} X + Y \qquad\qquad K_{eq} = k_1 / k_{-1}$$



G:F12-5

• Arrhenius proposed that rate constant varies with T as:

$$k = Ae^{(-\Delta G‡/RT)}$$

k = rate constant; A = frequency factor constant

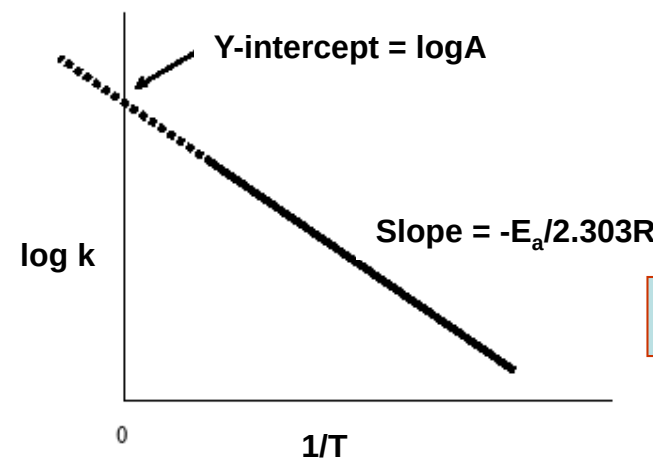
•Also used as: $k = Ae^{(-Ea/RT)}$ where E_a = energy of activation

•Another form: $\ln k = -E_a/RT + \ln A$ or

$\log_{10} k = -E_a/2.303RT + \log A$

Arrhenius Plots

• Plot of log k versus 1/T gives slope $-E_a/2.303R$ with + logA as Y intercept



T = deg K

(4) Allosteric Enzymes

- **Regulatory enzymes** are important control sites for metabolic pathways
  - Modulators bind and alter kinetics to produce non-Michaelis-Menten reaction profiles

- Aspartate transcarbamoylase (ATC)
  - two stacked catalytic clusters each with 3 catalytic polypeptides
  - Also 3 regulatory clusters each with 2 regulatory polypeptides
  - Modulator binding produces large changes in ATC conformation and activity

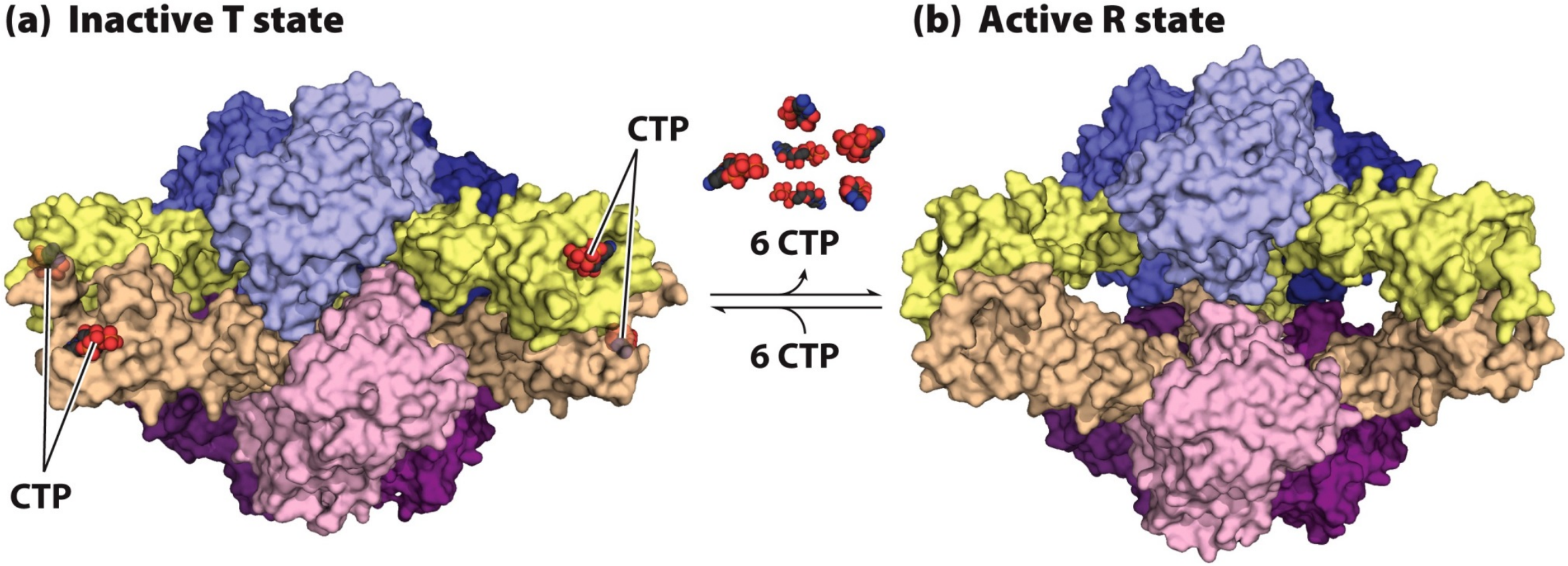


Figure 6-33
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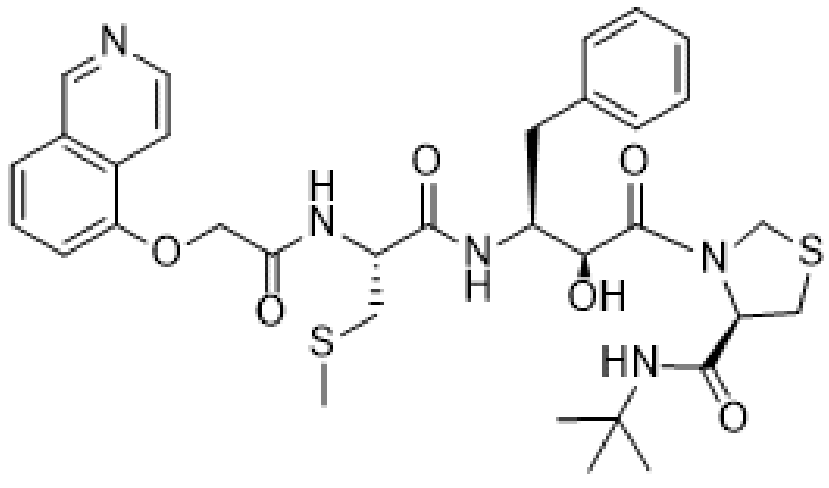
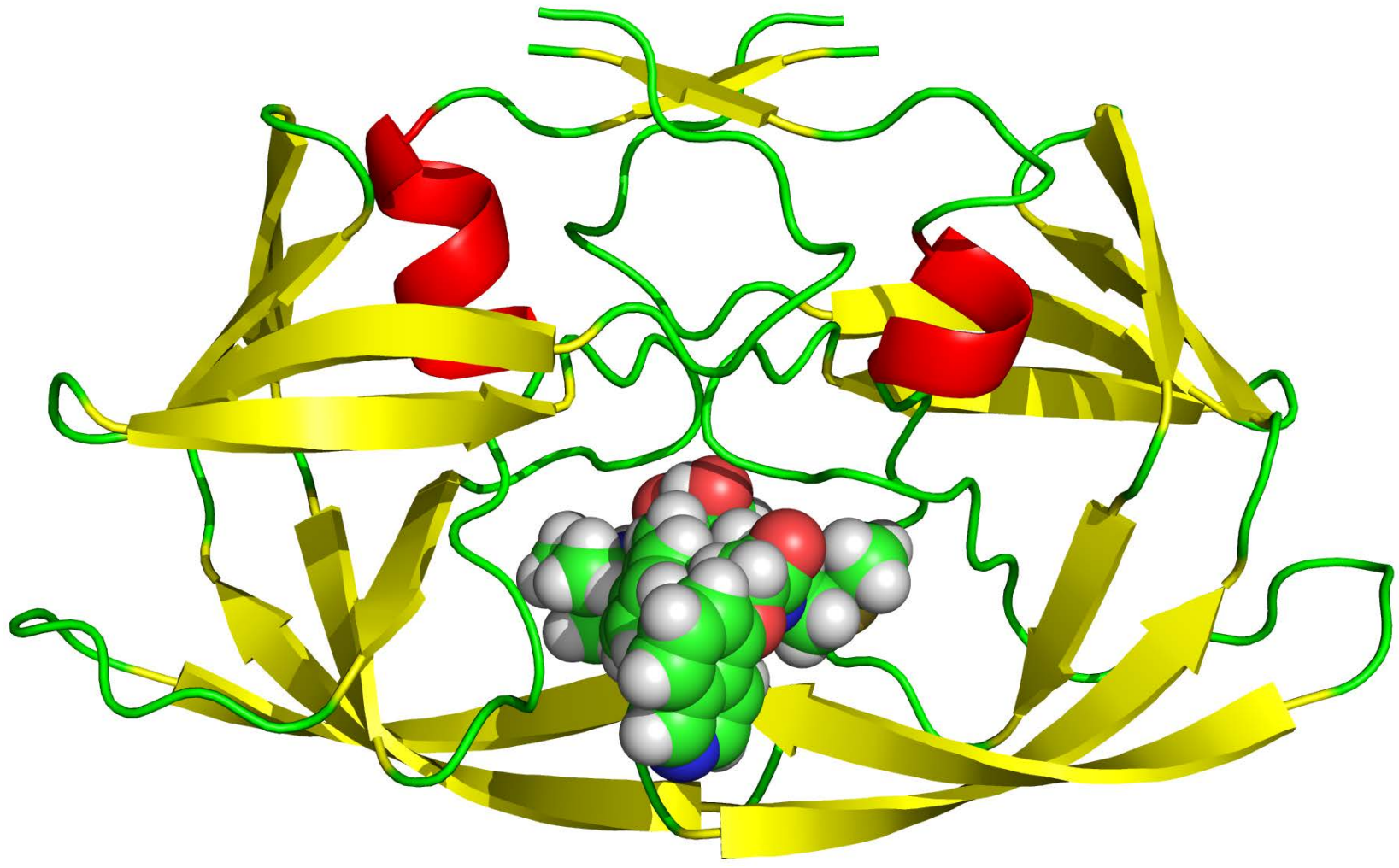
Enzymes can be regulated

– The regulation can be done by the following means:
  - Small molecules (competition, allostery)
  - Covalent modification (phosphorylation, ADP-ribosylation)
  - Degradation
  - Synthesis



Kynostatin-272
transition state inhibitor



Inhibition of HIV-1 protease by KNI-272

Noncovalent Modification: Allosteric Regulators

- Allosteric effectors or modulators are generally small chemicals
- Allosteric effectors can be positive, or improve enzymatic catalysis
- Allosteric effectors can be negative, or reduce enzymatic catalysis

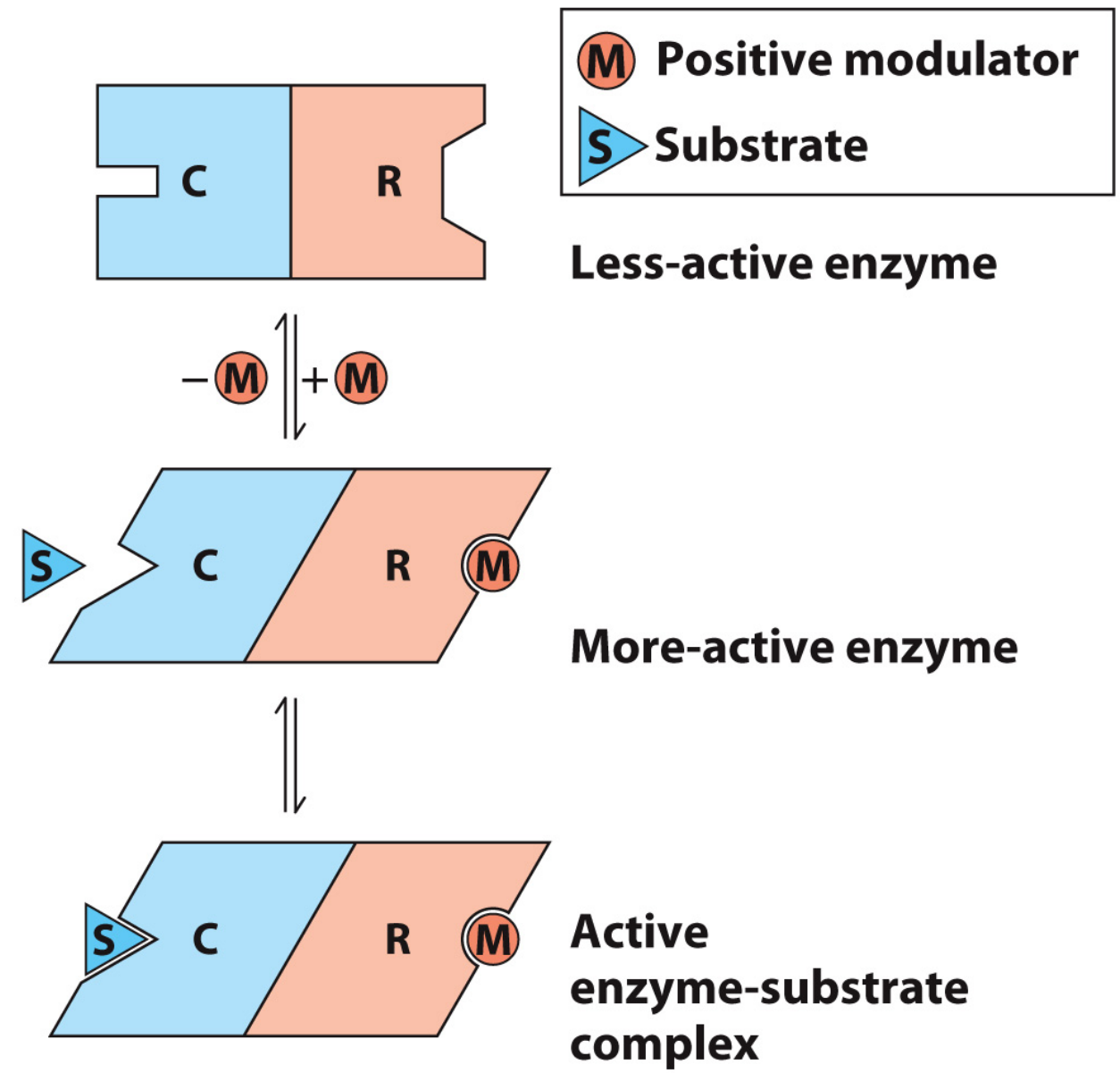


Figure 6-33
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Kinetics of Allosteric Enzymes

- many allosteric enzymes **do not obey** Michaelis-Menten kinetics
- plot of v_o vs [S] is not **hyperbolic** but **sigmoidal**

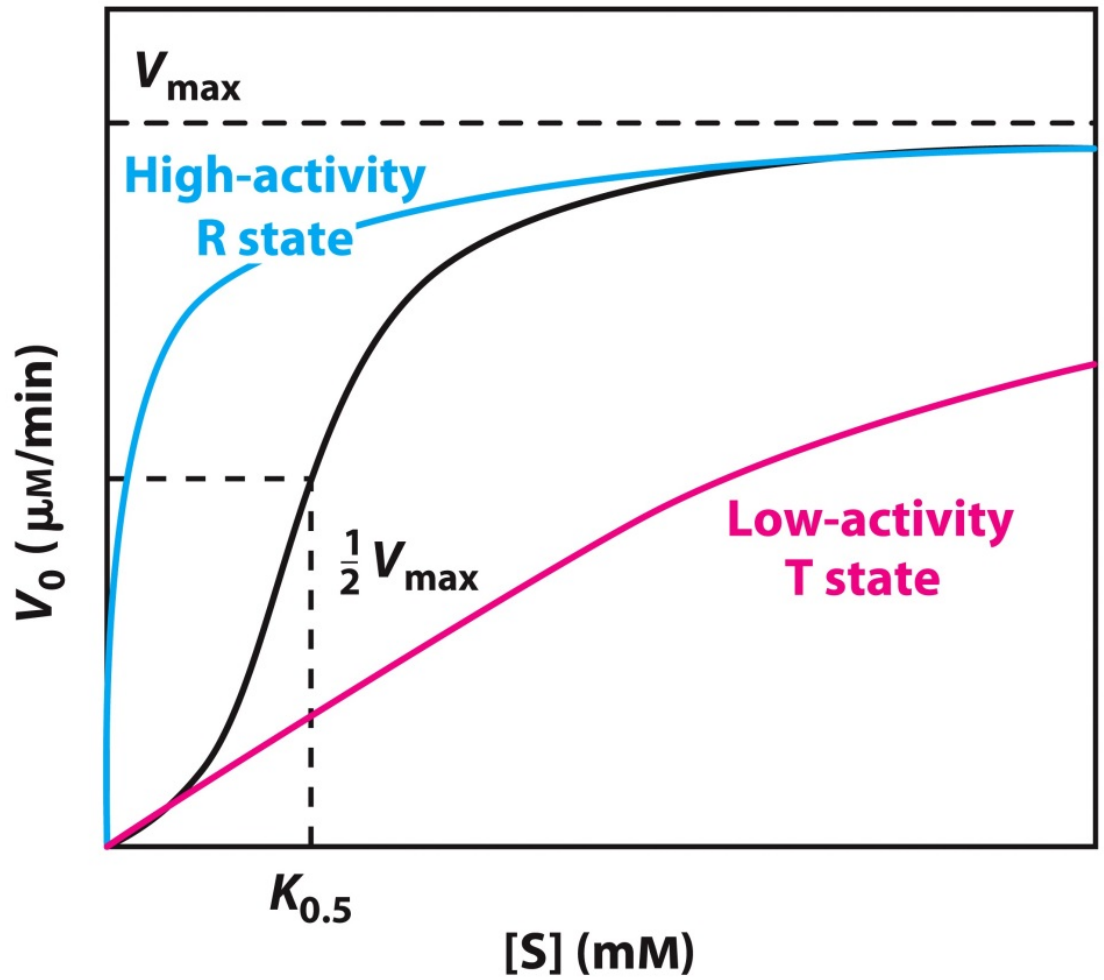

Figure 6-34a
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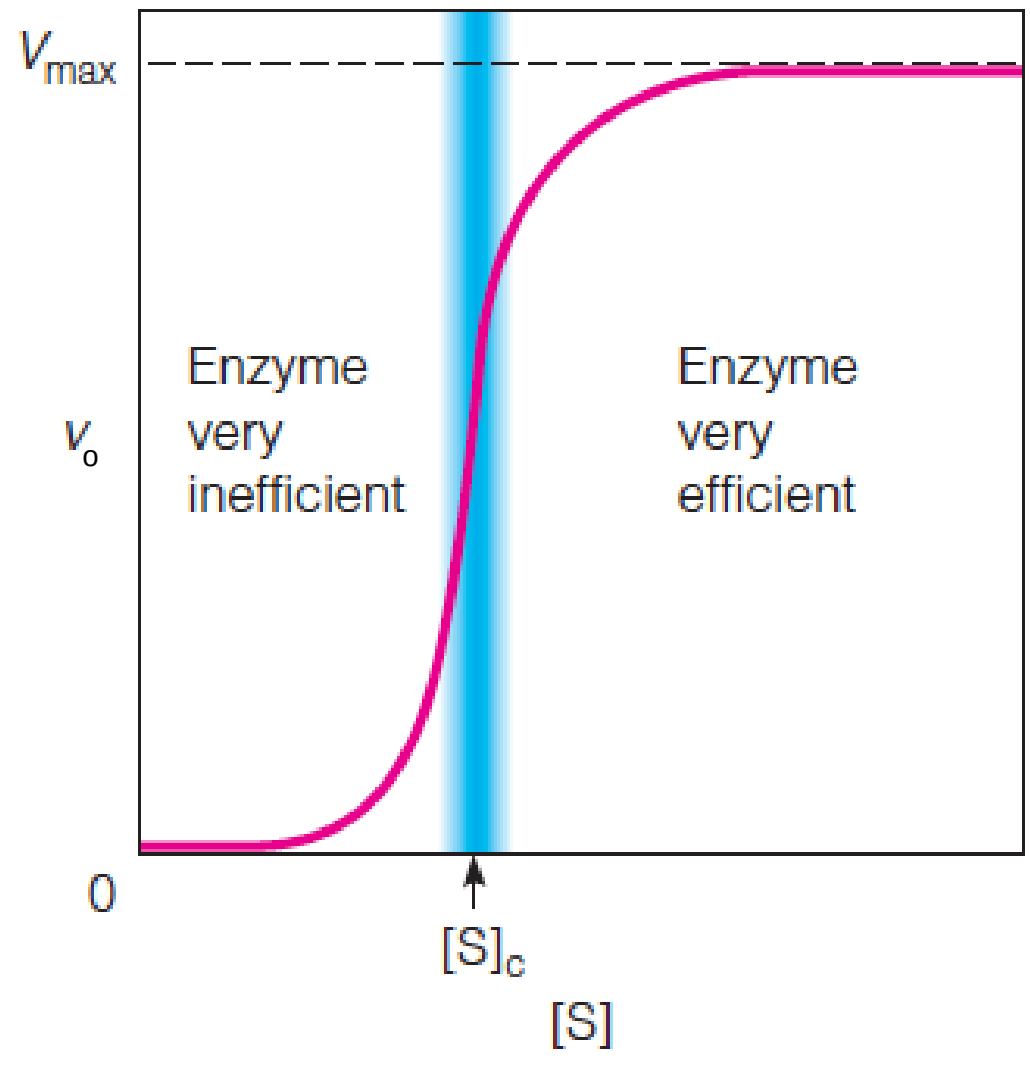
Occurs due to:

- **cooperative** substrate-binding
- binding of an **allosteric** modulator
- enzyme is **multi-subunit** and shows changes in disposition of subunits upon substrate/modulator binding
  - the $K_{0.5}$ replaces K_M

The Regulation of Enzyme Activity: Allosteric Enzymes

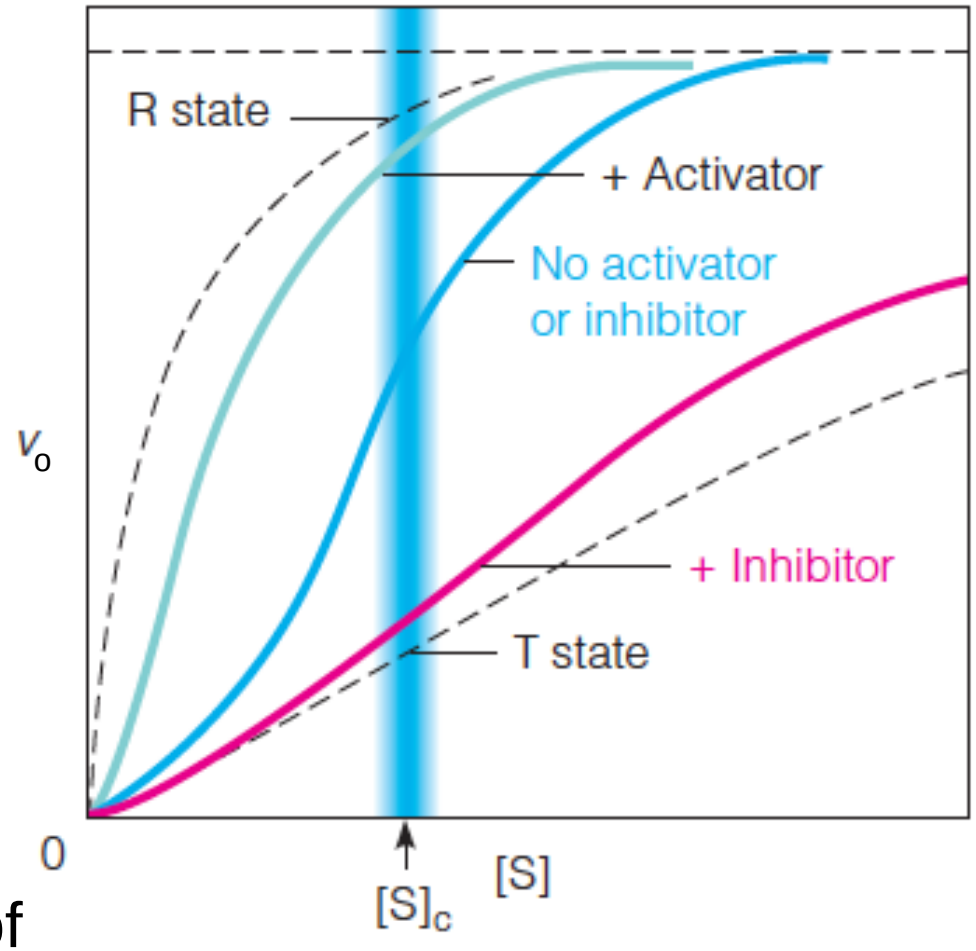
Effect of extreme homoallostery:

- The v_o vs. [S] curve is shown for a hypothetical enzyme with extreme positive cooperativity in substrate binding
- At concentrations below the enzyme is almost inactive; above this concentration, it is very active
- Substrate can accumulate to $[S]_c$ but at higher concentrations it will be processed rapidly
- The vertical blue line represents the homeostatic concentration range for substrate



The Regulation of Enzyme Activity: Allosteric Enzymes

- Allosteric enzymes show cooperative substrate binding and can respond to a variety of inhibitors and activators
- In the absence of activation or inhibitors, the v_o vs. [S] curve is sigmoidal
- ***Activators*** shift the system toward the ***R state***
- ***Inhibitors*** stabilize the ***T state***
- $[S]_c$ represents the homeostatic concentration range for S
- Note that effectors significantly alter the activity of the enzyme over this range of [S]



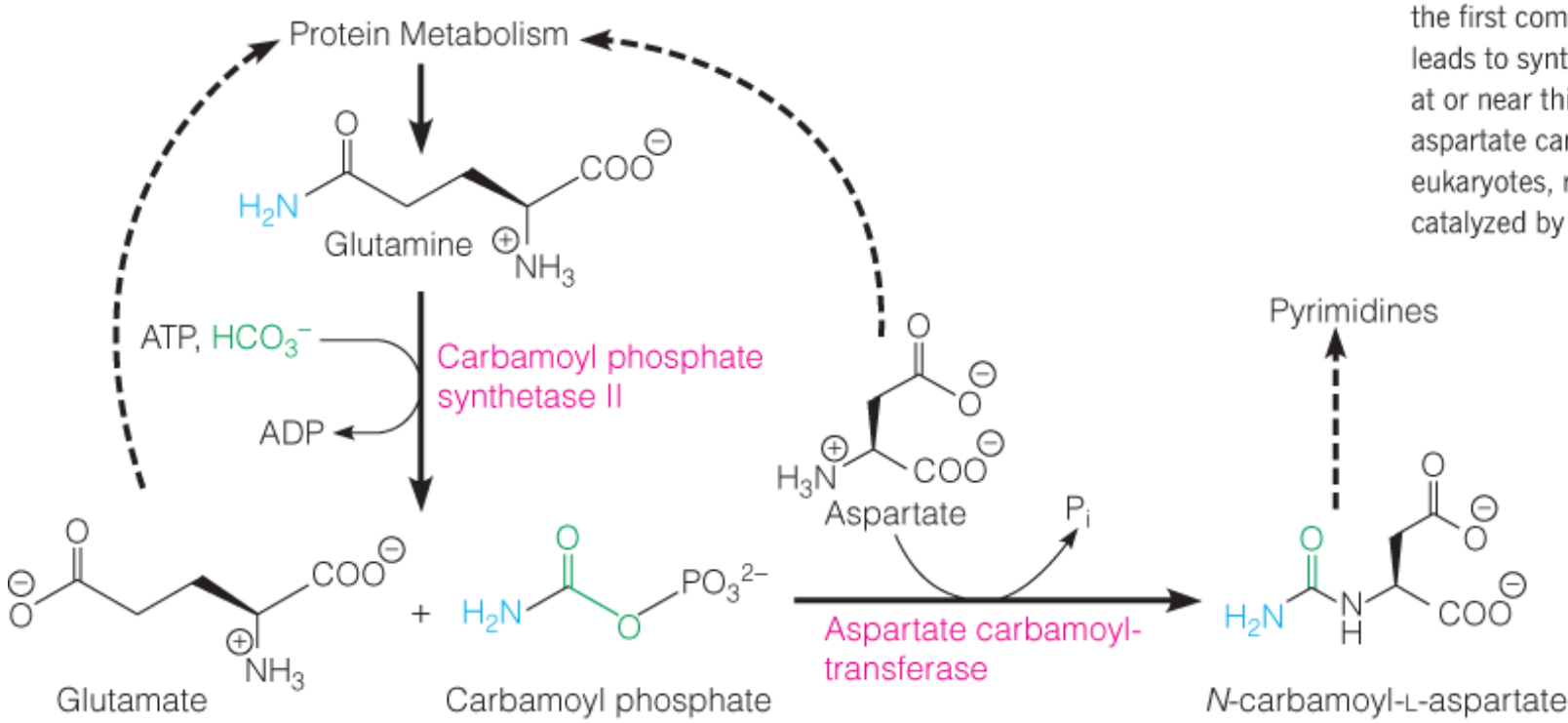
The Regulation of Enzyme Activity: Allosteric Enzymes

Control points in pyrimidine synthesis:

- The formation of ***N*-carbamoyl-L-aspartate** from carbamoyl phosphate and aspartate is the first committed step in synthesis of pyrimidine nucleotides
  - Control at this point is essential
- In prokaryotes, the aspartate carbamoyltransferase (ATCase) is regulated

FIGURE 11.47

Control points in pyrimidine synthesis. This figure shows the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and aspartate. This reaction is the first committed step in a series of reactions that leads to synthesis of pyrimidine nucleotides, so control at or near this point is essential. In prokaryotes, the aspartate carbamoyltransferase is regulated; in most eukaryotes, regulation is on the preceding step, catalyzed by carbamoyl phosphate synthetase II.



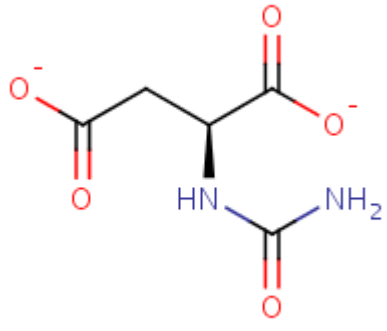
The Regulation of Enzyme Activity: Allosteric Enzymes

Regulation of ATCase by ATP and CTP:

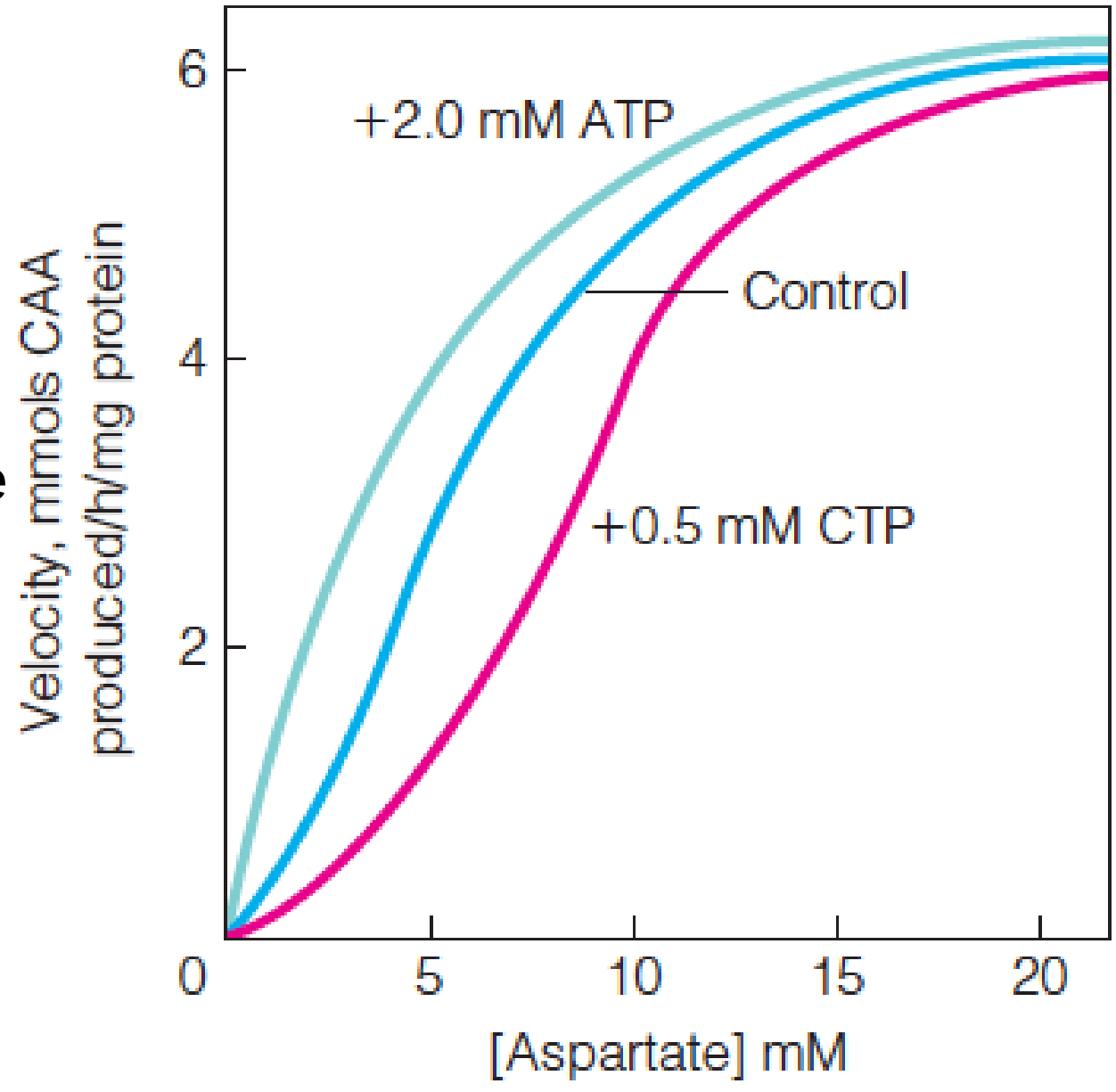
- **ATP is an activator.**
- **CTP is an inhibitor.**

•**control** shows the enzyme behavior in the **absence of both regulators**

•*N*-Carbamoyl-L-aspartate (CAA) is the product of the reaction.



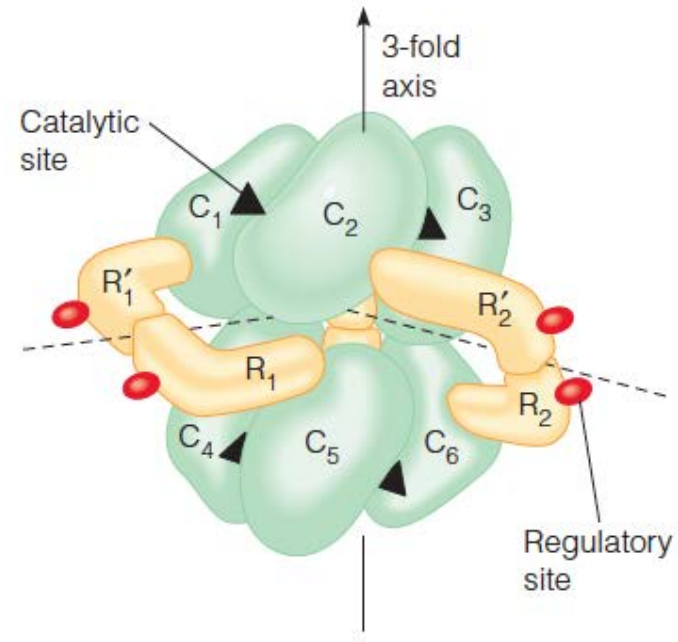
CAA



The Regulation of Enzyme Activity: Allosteric Enzymes

(a) Quaternary structure of ATCase in the T state.

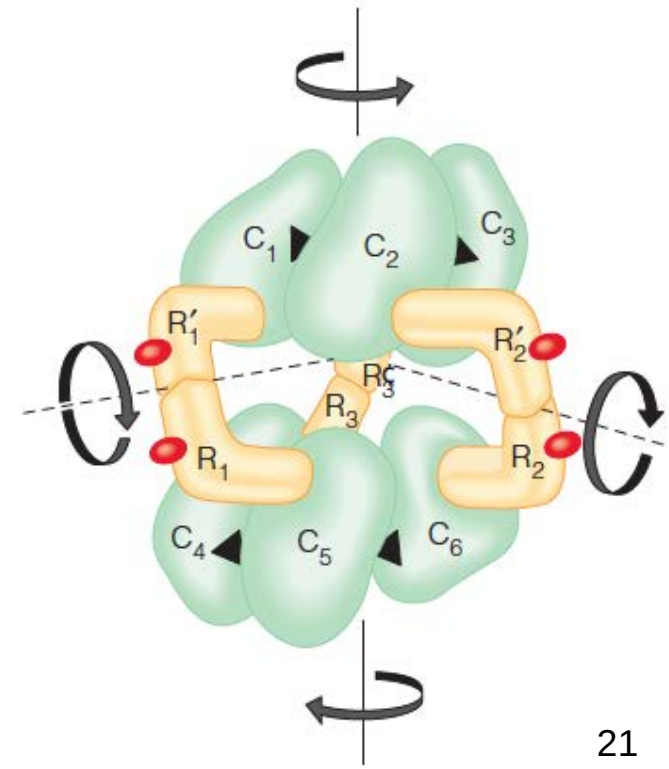
- The enzyme has the six catalytic subunits (C) and six regulatory subunits (R).
- Six catalytic sites lie in or near the grooves between the catalytic subunits.
- Regulatory sites lie on the outer surfaces of the regulatory subunits.



(a) ATCase: T state

(b) Transition of ATCase to the R state.

- The transition involves a rotation of the regulatory subunits, which pushes the two tiers of catalytic subunits apart and rotates them slightly about the three-fold axis



(b) ATCase: R state

Some Reversible Covalent Modifications

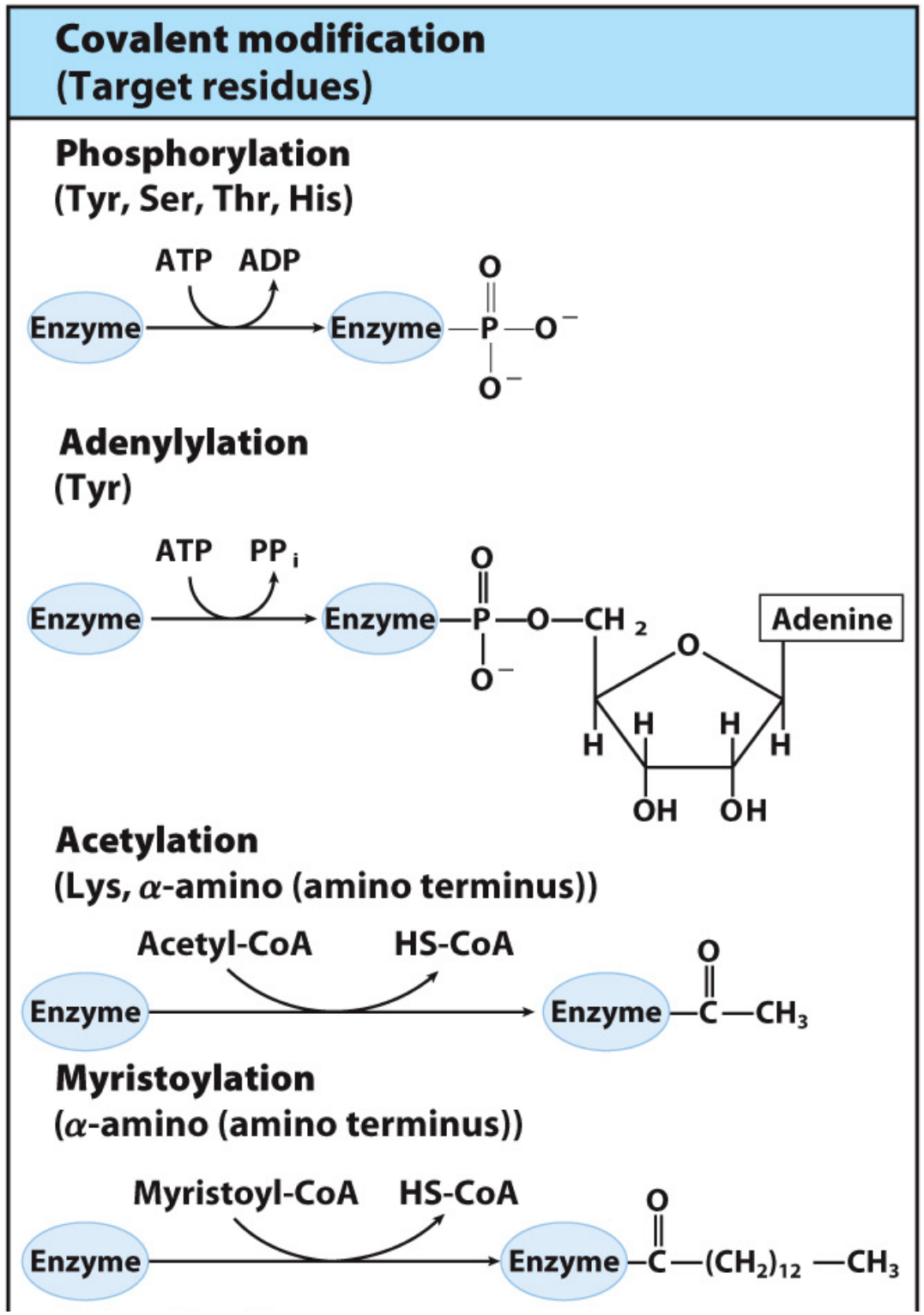


Figure 6-36

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Appendix: Derivation of Ionizable Equation

This derivation is taken from Voet and Voet, 2004, pp 492-493.

D. *The Michaelis–Menten Equation for Ionizable Enzymes—Equation [14.47]*

In the model presented in Section 14-4 to account for the effect of pH on enzymes, the dissociation constants for the ionizations are

$$K_{E2} = \frac{[H^+][E^-]}{[EH]} \qquad K_{ES2} = \frac{[H^+][ES^-]}{[ESH]}$$

$$K_{E1} = \frac{[H^+][EH]}{[EH_2^+]} \qquad K_{ES1} = \frac{[H^+][ESH]}{[ESH_2^+]} \qquad [14.A19]$$

Protonation and deprotonation are among the fastest known reactions, so that, with the exception of the few enzymes with extremely high turnover numbers, it can be reasonably assumed that all acid–base reactions are at equilibrium. The conservation condition is

$$[E]_T = [EH]_T + [ESH]_T \qquad [14.A20]$$

where $[E]_T$ is the total enzyme present in any form,

$$[EH]_T = [EH_2^+] + [EH] + [E^-]$$
$$= [EH]\left(\frac{[H^+]}{K_{E1}} + 1 + \frac{K_{E2}}{[H^+]}\right)$$
$$= [EH]f_1 \qquad [14.A21]$$

and

$$[ESH]_T = [ESH_2^+] + [ESH] + [ES^-]$$
$$= [ESH]\left(\frac{[H^+]}{K_{ES1}} + 1 + \frac{K_{ES2}}{[H^+]}\right)$$
$$= [ESH]f_2 \qquad [14.A22]$$

Then making the steady-state assumption

$$\frac{d[ESH]}{dt} = k_1[EH][S] - (k_{-1} + k_2)[ESH] = 0 \qquad [14.A23]$$

and solving for [EH]

$$[EH] = \frac{(k_{-1} + k_2)[ESH]}{k_1[S]} = \frac{K_M[ESH]}{[S]} \qquad [14.A24]$$

Therefore, from Eq. [14.A21],

$$[EH]_T = \frac{K_M[ESH]f_1}{[S]} \qquad [14.A25]$$

which, together with Eqs. [14.A20] and [14.A22], yields

$$[E]_T = [ESH]\left(\frac{K_M f_1}{[S]} + f_2\right) \qquad [14.A26]$$

As in the simple Michaelis–Menten derivation, the initial rate is

$$v_o = k_2[ESH] = \frac{k_2[E]_T}{\left(\frac{K_M f_1}{[S]}\right) + f_2} = \frac{(k_2/f_2)[E]_T[S]}{K_M(f_1/f_2) + [S]} \qquad [14.A27]$$

Then defining the "apparent" values of K_M and $V_{max} = k_2[E]_T$ at a given pH:

$$K'_M = K_M(f_1/f_2) \qquad [14.A28]$$

and

$$V'_{max} = V_{max}/f_2 \qquad [14.A29]$$

the Michaelis–Menten equation modified to account for pH effects is

$$v_o = \frac{V'_{max}[S]}{K'_M + [S]} \qquad [14.47]$$