BS11  Final Exam 1997  Answer Key

Question 1. (14 pts) Protein kinase A (PKA) was examined by gel filtration at two protein concentrations: 0.1 nM and 10 nM. The same total amount of protein was run in both cases. The results at both concentrations are shown below on the same elution profile.

<table>
<thead>
<tr>
<th>Relative molecular sizes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A : B : C</td>
</tr>
<tr>
<td>1.8 : 1.0 : 0.4</td>
</tr>
</tbody>
</table>

(6) A). Explain the results by labeling the peaks to indicate their composition.

A: \( R_2C_2 \)  
B: \( R_2 \)  
C: \( C \)

(4) B). Suggest an experiment that would support your conclusions in part A.

Ans: Analyze column fractions for cAMP binding activity and for protein kinase activity. A should have both activities, B should bind cAMP only and C should have protein kinase activity only.

(4) C). Explain why the difference in concentration resulted in the different elution profiles.

Ans: \( R_2C_2 \Leftrightarrow R_2 + 2C \)  
The Kd for tetramer formation must be between 0.1 nM and 10 nM.

Question 2. (22 pts) Consider the reaction:

\[
E + S \quad \Leftrightarrow \quad ES \quad \rightarrow \quad E + P
\]

(2) A) Write the expression for the velocity of the enzyme catalyzed reaction in terms of \( E_{tot} \), \( K_m \) and \( S \).

Ans: \( v = \frac{k_2[E_{tot}][S]}{(K_m + [S])} \)
(2) **B)** Draw a curve of \( v \) versus (\( S \)) and show on the graph the significance of \( \text{Km} \) and \( k_2 \).

**Ans:**

![Graph of v vs S showing Km and k2](image)

(2) **C)** Write \( \text{Km} \) in terms of the kinetic constants.

**Ans:** \( \text{Km} = \frac{(k_{-1} + k_2)}{k_1} \)

(2) **D)** What is the order of the reaction (with respect to \( S \)) (i) when the enzyme is saturated _0_ and (ii) when the enzyme operates at low (\( S \)) _first_.

(2) **E)** What are the units of \( \text{Km} \) _M_, \( k_1 \) _M\(^{-1}\)s\(^{-1}\_), \( k_{-1} \) _s\(^{-1}\_), and \( k_2 \) _s\(^{-1}\__.

(2) **F)** Under what conditions is \( \text{Km} \) equal to the dissociation constant of the enzyme-substrate complex.

**Ans:** \( \text{Km} = K_s = \frac{k_{-1}}{k_1} \) when \( k_{-1} \gg k_2 \)

(2) **G)** What is the significance of \( \frac{k_2}{\text{Km}} \). What is the upper limit of the value of \( \frac{k_2}{\text{Km}} \).

**Ans:** \( \frac{k_2}{\text{Km}} \) is the specificity constant that describes the reaction of free enzyme and substrate. It is the effective second order rate constant for the reaction. The upper limit of \( \frac{k_2}{\text{Km}} \) is the rate constant of a diffusion-limited reaction: \( 10^8 - 10^9 \) M\(^{-1}\)s\(^{-1}\).

(2) **H)** What does the \( v \) versus (\( S \)) curve look like when an uncompetitive inhibitor is added to the reaction.

**Ans:**

![Graph of 1/v vs 1/[S] with [I] = 0 and [I] = X](image)
4) I) A substance, I, is a competitive inhibitor of the enzyme. How would one determine the value of Ki? Show what data are required, how they would be used, and how one would obtain the value of Ki.

Ans: Measure v as a function of [S], plus and minus I. Determine Km and Km_{app} by plotting 1/v vs. 1/[S]. Use the equation Km_{app} = Km(1 + ([I]/Ki)) and solve for Ki.

2) J) Write the Haldane equation and state its significance.

Ans: Keq = \( \frac{k_{cat}/K_m}{(k_{cat}/K_m)_r} \) All reactions reach an equilibrium dependent on the ratio of rate constants, not the [E] or the [S]. Values of Km's and kcat's can vary so long as the ratio remains the same.

Question 3. (9 pts)

3) A). ΔG° for the hydrolysis of the phosphoanhydride bonds of ATP is -7.5 kcal/mol, while that for the hydrolysis of phosphate monoesters is -3.5 kcal/mol. Indicate two reasons for the greater free energy content (more negative free energy of hydrolysis) of ATP.

Ans: 1 - Electrostatic repulsion between the charged groups of ATP compared to ADP and Pi. 2 - Resonance stabilization of ADP and Pi compared to ATP (increase in entropy). 3 - Greater solvation of ADP and Pi compared to ATP.

3) B). Both ATP and phosphate monoesters are equally stable compounds in water. How is this compatible with the values of ΔG° given above.

Ans: There is no necessary relationship between ΔG and the rate of a reaction.

3) C). Why is phosphate and not sulfate used to link the nucleosides in RNA and DNA.

Ans: Phosphate is tribasic; it can link two molecules and still remain negatively charged. Sulfate is only dibasic; no negative charge remains when it links two molecules. The negative charge of a phosphodiester bond is important in decreasing the rate of cleavage because it protects against attack by OH⁻.
**Question 4. (11 pts)** Hemagglutinin (HA), a coat protein of the influenza virus important for entry of the virus into the cell, contains a conserved 28 amino acid sequence that is critical for fusion of the viral membrane with the cell’s endosome after endocytosis of the virus. The sequence of a 28mer peptide corresponding to this conserved region is:

1- IEKTNEKFHQIEKEFSEVEGRIQDLEKY-28 and has a MW = 4,500.

The conformation of the peptide in water at pH 4.8 and 7.2 was examined by circular dichroism (CD) and the results are shown in the Figure. Very briefly, circular dichroism allows for an analysis of a peptide’s secondary structure by measuring the unequal absorption of left- and right-circularly polarized light as it passes over alpha-helices, beta-structures or random coils. Peptides with beta-structure have a strong absorbance at 170nm in the negative y-axis direction and a strong absorbance at 200nm in the positive y-axis direction. The characteristic absorptions of all three types are summarized below.

<table>
<thead>
<tr>
<th>Wavelength, (nm)</th>
<th>Closed Circles = pH 4.8</th>
<th>Open Circles = pH 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>170nm, negative</td>
<td>200nm, positive</td>
</tr>
<tr>
<td>220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>260</td>
<td></td>
<td></td>
</tr>
<tr>
<td>280</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2° Structure | Absorbance
beta-structure: 170nm, negative 200nm, positive
alpha-structure: 208nm, 222nm negative 192nm, positive
random coil: 200nm, negative

(4) A. Indicate the most likely structure of the peptide at pH 4.8 alpha helix and 7.2 random coil

The effect of pH on the tertiary and quaternary structure of the peptide was studied by sedimentation equilibrium measurements. These indicated that the average molecular weight of the peptide at pH 4.8 is 13,900 and at pH 7.2 it is 5,000.

(3) B. Show in as much detail as possible the quaternary structure of the peptide at pH 4.8 and 7.2. Ans: at pH 7.2: monomeric random coils

at pH 4.8: bundle of three alpha-helices (triple coiled coil)
A version of the 28-mer peptide was synthesized with the normal Asp and Glu residues changed to Asn and Gln. The CD spectrum of this mutant peptide at pH 7.2 was identical to that of the normal peptide at pH 4.8. Furthermore, the mutant peptide had a molecular weight of 14,000 at pH 7.2. Finally, a recombinant virus expressing a HA with the Asn and Gln mutations was able to fuse with the host cell directly at the plasma membrane.

(4) C. Explain the effect of the mutations on the structure of the peptide.

Ans: The negative charged residues (D and E) prevent formation of the alpha-helix; when the charge is neutralized either by protonation at pH 4.8 or by mutation to N and Q, then the alpha-helices form. The helices can then form the triple helix which promotes fusion.

Question 5. (36 points total over three parts)
Part I. (10 pts)
(6) A. Name the labeled structures in the mitotic spindle shown in the Figure.

(See figure for this question on the copy of the exam in your sourcebook.)

A). kinetochore
B). chromosome (sister chromatids)
C). kinetochore microtubules
D). centrosome or centrioles
E). astral microtubules
F). polar microtubules

(1) B). Indicate on the figure the + end and the - end of a microtubule. Ans: the plus end is away from the centrosomes, the minus end is at the centrosomes.

(3) C). Indicate how one could determine whether or not the microtubules in the spindle are in a dynamic state. Ans: 1 - Photobleach a section of MTs labeled with a fluorescent molecule. Watch for movement. (Or use a caged-fluorescent molecule and activate with a flash.) 2 - Add a drug, such as colchicine, that inhibits polymerization of MTs and watch to see if MTs disappear.
Part II. (10 pts) **Discodermolide**, a natural marine product, causes cell cycle arrest in human and murine cell lines. The effect of this compound on microtubules was determined by the following experiments.

(3) A). In one experiment, cells were treated with 20 nM [³H]discodermolide and then were lysed in a non-ionic detergent in the presence and absence of colchicine. The lysates were separated by centrifugation into a supernatant solution containing cytoplasmic proteins and a pellet with nuclei and other cell structures. In a second experiment, cells were lysed first, then treated with [³H]discodermolide in the presence and absence of taxol, and then separated by centrifugation into supernatant and pellet. (Lysis of cells in the absence of any compound leads to the breakdown of microtubules.) The amount of [³H]discodermolide that is bound to protein in either the supernatant or the pellet is shown in the figures below as a percentage of the total [³H]discodermolide added.

\[ -/+ \text{Colchicine} \quad -/+ \text{Taxol} \]

(See all of the figures for this question on the copy of the exam in your sourcebook.)

Discodermolide binds preferentially to:

a). free tubulin dimers  
b). colchicine-bound tubulin dimers  
c). taxol-bound tubulin dimers  

\[=>\] d). free microtubules  
e). colchicine-bound microtubules  
f). taxol-bound microtubules

(3) B). Tubulin dimers (10 µM) were incubated with taxol (10 µM) and 1 mM GTP. Then [³H]discodermolide at several concentrations was added, and after 30 minutes, the solutions were separated into pellet and supernatant fractions. The radioactivity in the pellets was measured and is reported as the percentage of the maximum amount of radioactivity in the pellet.

<table>
<thead>
<tr>
<th>[disco] (µM)</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>% saturation</td>
<td>11.3</td>
<td>56.4</td>
<td>99.4</td>
<td>99.7</td>
<td>100</td>
</tr>
</tbody>
</table>

Indicate the stoichiometry of the interaction between tubulin dimers and discodermolide.

Ans: 1:1.
C. Tubulin dimers (10 $\mu$M) and 1 mM GTP were incubated with $[^{3}H]$taxol. A competition experiment was then carried out by adding various concentrations of nonradioactive taxol or discodermolide; after 30 minutes, the solutions were separated into pellet and supernatant fractions. The radioactivity in the pellet was measured and is reported as percentage of the radioactivity in the pellet in the absence of cold taxol or discodermolide.

(Fig.)

The relative affinities of taxol and discodermolide for tubulin dimers are: (Ka is an association constant)

a). $K_a$(disco) < $K_a$(taxol)
b). $K_a$(disco) = $K_a$(taxol)

$\Rightarrow$ c). $K_a$(disco) > $K_a$(taxol)

Part III. (16 pts) The effect of discodermolide on the cell cycle was examined as follows. A suspension of fertilized clam eggs in the presence of $[^{35}S]$methionine was placed in two tubes; to one of them 10 nM discodermolide was added. Samples were taken from both tubes as a function of time and analyzed for the amount of M-phase cyclin in the eggs and for the presence of mitotic chromosomes. The results are shown below.

(Fig.)

To try to understand the effect of discodermolide on cyclin behavior, the experiment was repeated in the presence of the protein synthesis inhibitor emetine added just before the cells enter mitosis. The results are shown below. In the absence of discodermolide, the cells entered and exited mitosis normally and divided into two daughter cells. In the presence of discodermolide, the cells stayed in mitosis for 2 hours and then decondensed their chromosomes and reformed nuclei without dividing.

(Fig.)

A. Discodermolide has the following effect on cyclin synthesis:

a). increase
b). partial decrease
c). complete inhibition

$\Rightarrow$ d). no effect
(4) B). Discodermolide has the following effect on cyclin degradation:
   a). increase
   => b). partial decrease
   c). complete inhibition
   d). no effect

(4) C). Suggest a mechanism for the arrest caused by discodermolide. Indicate in which phase of the cycle arrest is likely to occur.

   Ans: Discodermolide stabilizes the structure of MTs; it prevents their dynamic reshuffling to form spindle MTs which capture and align and separate chromosomes. Cells won't move beyond prometaphase (arrest before metaphase.)


   Ans: Since discodermolide does not completely inhibit cyclin degradation, the absence of cyclin synthesis means that the cyclin will eventually be destroyed. With no cyclin, MPF activity is lost and the cells exit mitosis.

(5) Question 6. Match the following scientists to their principal contribution in the fields of biochemistry and cell biology.

   6 A). Konrad Bloch  1). chemistry of phosphates
   2 B). Christian Bohr  2). effect of pH on O₂ affinity of hemoglobin
   10 C). Linus Pauling  3). cooperative behavior of hemoglobin
   1 D). Frank Westheimer  4). structure of rough ER
   5 E). Maude Menten  5). kinetic analysis of enzymes

   6). pathway for cholesterol synthesis
   7). citric acid cycle
   8). pathway of glycolysis
   9). protonmotive force
   10). structure of alpha helix
**Question 7. (15 pts)** Skeletal muscle of a crocodile takes up glucose from the blood during a meal. The glucose is to be stored as glycogen. Some of the glucose is used in glycolysis to supply the energy for the conversion of the remaining glucose to glycogen.

(3) **A).** How many ATP equivalents are required to add one molecule of glucose to glycogen? \(2\)

(3) **B).** How many ATP equivalents are produced by the glycolytic degradation of one molecule of free glucose? \(2\)

(3) **C).** Write the complete stoichiometric reaction for the glycolysis of glucose.

\[
\text{Ans: } \text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{Pi} \longrightarrow 2 \text{Pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O}
\]

or

\[
\text{Glucose} + 2\text{ADP} + 2\text{Pi} \longrightarrow 2 \text{Lactate} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O}
\]

(3) **D).** What fraction of glucose does crocodile muscle convert to glycogen? \(1/2\)

(3) **E).** What fraction of glucose would be converted to glycogen if glucose could undergo complete oxidation to CO\(_2\)?

\[
\text{Ans: if 30 ATP/glucose: then 15/16}
\]

\[
\text{if 36 ATP/glucose: then 16/17}
\]

or about 0.94

**Question 8. (8 pts)** Choose one answer

(4) **A).** For Enzyme A, the value of \(k_{cat}/K_m\) is constrained to \(10^6 \text{M}^{-1} \text{sec}^{-1}\) and \([S] = 10^{-3} \text{M}\). The moles of product produced per mole of enzyme per second:

\[
\Rightarrow \text{a) increase as } K_m \text{ increases}
\]

\[
\text{b) decrease as } K_m \text{ increases}
\]

\[
\text{c) are independent of } K_m
\]

\[
\text{d) cannot be determined by available data}
\]
(4) **B).** Enzyme B catalyzes the reaction:

\[ E + S \rightleftharpoons ES \rightarrow E + P \]

For one substrate, S1, the Km is $10^{-5}$ M; for another substrate, S2, the Km is $10^{-7}$ M.

Which of the following statements is true:

a) S1 binds more tightly to E than does S2.

b) S2 binds more tightly to E than does S1.

c) S1 and S2 have the same affinity for E.

--> d) No conclusions can be made.

**Question 9. (12 pts)** In Hemoglobin Radcliffe, residue 99 $\beta$ Asp in the $\alpha_1\beta_2$ interface is changed to Ala; this hemoglobin has a higher $O_2$ affinity than does normal hemoglobin. Hemoglobin Kansas has a lower $O_2$ affinity than normal hemoglobin; residue 102 $\beta$ Asn in the $\alpha_1\beta_2$ interface is mutated to Thr.

(4) **A).** Which form of the molecule, oxy or deoxy, is stabilized by the mutations?

Radcliffe: **oxy**  Kansas: **deoxy**

(4) **B).** What is the value of n (the Hill coefficient) for normal hemoglobin? **2.8-3.0**

Would you expect the value of n for each abnormal Hb to be greater than, less than or equal to that of normal Hb?  Radcliffe: **less**  Kansas: **less**

(4) **C).** Draw the $O_2$ saturation curves of the mutant hemoglobins on the graph below which shows the curve for normal hemoglobin. (Label them clearly!)
**Question 10. (14 pts)** Aldehyde dehydrogenase catalyzes the reaction:

$$
\text{R}--\text{CH} + \text{NAD}^+ + \text{H}_2\text{O} \rightleftharpoons \text{R}--\text{C}--\text{O}^- + \text{NADH} + 2\text{H}^+
$$

Using fast measurements techniques, the following rate profile was obtained for the reaction of 1 pmole of enzyme with propionaldehyde and NAD$^+$:

![Rate profile diagram]

(6) **A).** Write the simplest kinetic scheme (sequence of reactions) to explain the results. Assume that NAD$^+$ binds to the enzyme before the aldehyde, and that either NADH or the carboxylic acid is released first from the enzyme.

**Ans:**

\[
\begin{align*}
\text{E} + \text{NAD}^+ & \rightarrow \text{E.NAD}^+ \\
\text{E.NAD}^+ + \text{RCOH} & \rightarrow \text{E.NAD}^+ \cdot \text{RCOH} \\
\text{E.NAD}^+ \cdot \text{RCOH} & \rightarrow \text{E.RCOO}^- \cdot \text{NADH} \\
\text{E} + \text{RCOO}^- & \rightarrow \text{E.RCOO}^- + \text{NADH}
\end{align*}
\]

(8) **B).** Iodoacetate reacts with only one cysteine residue of the enzyme and inactivates the enzyme. X-ray crystallographic analysis shows that this cysteine residue is in the active site of the enzyme. In other experiments, it was found that the rate of the enzymatic reaction increases and that the burst phase shown in the Figure disappears as the pH of the solution is increased.

Draw two diagrams showing the active site of this enzyme, one before and one after the reduction of NAD$^+$.

**Ans:**

![Diagrams of enzyme active site before and after reduction of NAD$^+$]
**Question 11. (16 pts)** A yeast protein, X, binds GTP and is involved in signal transduction. A mutant allele of the gene for X, $X^{\text{mut}}$, causes the signaling pathway to be constitutively "on". This mutation is suppressed by a mutation ($Y^{\text{sup}}$) in the gene for another protein, Y, so that yeast with the genotype $X^{\text{mut}}Y^{\text{sup}}$ appear to have normal signal transduction. When protein X is incubated with GTP in the presence of protein Y, phosphate appears in the buffer.

(4) **A).** What is the likely role of protein Y?

Ans: GAP - promotes hydrolysis of GTP by X

(4) **B).** What is the defect in the mutant form of X?

Ans: Either it binds but does not hydrolyze (or hydrolyzes only very slowly) GTP or it does not bind Y.

Cell lysates from wild-type cells are depleted of nucleotides and then incubated with either GDP, GTP, or GTP-$\gamma$-S, a non-hydrolyzable analog of GTP. The lysate is then immunoprecipitated with antibodies to protein Y attached to beads. The amount of protein X in the precipitate is quantitated:

<table>
<thead>
<tr>
<th>Amount of protein X in precipitate (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP</td>
</tr>
<tr>
<td>GTP</td>
</tr>
<tr>
<td>GTP-$\gamma$-S</td>
</tr>
</tbody>
</table>

(4) **C).** Why is more protein X in the precipitate with GTP as compared to GDP?

Ans: Y binds preferentially to the GTP-bound form of X

(4) **D).** Why is more protein X in the precipitate with GTP-$\gamma$-S as compared to GTP?

Ans: X is trapped in its GTP-bound conformation since it can’t hydrolyze the GTP-$\gamma$-S, and so forms a stable complex with Y.
**Question 12. (14 pts)** Mevalonate is synthesized from β-OH-methylglutaryl-CoA (HMG-CoA) in a reaction catalyzed by HMG-CoA reductase:

\[
\text{HO-CH}_3\text{O} - \text{S-CoA} + \text{NADPH + H}^+ \rightleftharpoons \text{Intermediate + NADPH + H}^+ \rightleftharpoons \text{HO-CH}_3\text{H-H} - \text{OH} \text{CoASH}
\]

(4) A). Write the structure of the intermediate.

**Ans:**

\[
\text{HO-CH}_3\text{O} - \text{H} \text{COO}^-
\]

(4) B). Incubation of the intermediate with the enzyme in the presence of 4-[\textsuperscript{3}H]NADPH results in the formation of the following compound.

Is the \textsuperscript{3}H in the proR or proS position? **proR**

(6) C). Determine the standard free energy change for the overall reaction:

\[
\text{HMG-SCoA + 2 NADPH + 2H}^+ \rightarrow \text{Mevalonate + CoASH + 2NADP}^+
\]

The standard redox potentials for the half reactions are:

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( E_o' ) (volt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{HMG-SCoA + 2H}^+ + 2e^- \rightarrow [I] + \text{CoASH} )</td>
<td>-0.41</td>
</tr>
<tr>
<td>( [I] + 2H^+ + 2e^- \rightarrow \text{Mevalonate} )</td>
<td>-0.16</td>
</tr>
<tr>
<td>( \text{NADP}^+ + H^+ + 2e^- \rightarrow \text{NADPH} )</td>
<td>-0.32</td>
</tr>
</tbody>
</table>

\( F = 23 \text{ kcal/volt mol} \)

**Ans:**

\[
\Delta G^{o'} = -nF \Delta E_o' = -2(23\text{ kcal/V·mol})[-0.41+.32]V + (2)(23\text{ kcal/V·mol})[-0.16+.32]V
\]
\[
= 4.14 \text{ kcal/mol} - 7.36 \text{ kcal/mol} = -3.2 \text{ kcal/mol}
\]

**OR**

\[
\Delta G^{o'} = -2(23\text{ kcal/V·mol})[-0.41 - 0.16 + 2(0.32)]V
\]
\[
= -2(23\text{ kcal/V·mol})(-0.07V) = -3.2 \text{ kcal/mol}
\]
Question 13. (24 pts)

(4) A). The rate of hydrolysis of ATP by myosin (in the absence of any actin), measured by phosphate release, is limited by:

1). the rate of binding of ATP
2). the rate of the hydrolytic step

=> 3). the rate of release of Pi
4). the rate of release of ADP
5). the rate of interaction between actin and myosin

(4) B). The rate of hydrolysis of ATP by one myosin in contact with an actin filament is limited by:

1). the rate of binding of ATP
2). the rate of the hydrolytic step

=> 3). the rate of release of Pi
4). the rate of release of ADP
5). the rate of interaction between actin and myosin

(4) C). The rate of movement of an actin filament propelled by 100 myosin molecules in the presence of ATP is limited by:

1). the rate of binding of ATP
2). the rate of the hydrolytic step
3). the rate of release of Pi

=> 4). the rate of release of ADP
5). the rate of interaction between actin and myosin

(4) D). The rate of hydrolysis of GTP by 100 G-proteins, measured by phosphate release, is limited by:

1). the rate of binding of GTP
2). the rate of the hydrolytic step
3). the rate of release of Pi

=> 4). the rate of release of GDP
5). the rate of interaction between receptor and G-protein
(4) E). In a cell membrane, the rate of hydrolysis of GTP by 100 G-proteins in the presence of 1 ligand-bound receptor is limited by:

1). the rate of binding of GTP
2). the rate of the hydrolytic step
3). the rate of release of Pi
4). the rate of release of GDP

==> 5). the rate of interaction between receptor and G-protein

(4) F). In a cell membrane, the rate of hydrolysis of GTP by 100 G-proteins in the presence of 100 ligand-bound receptors is limited by:

1). the rate of binding of GTP

==> 2). the rate of the hydrolytic step
3). the rate of release of Pi
4). the rate of release of GDP
5). the rate of interaction between receptor and G-protein