Phosphoryl Transfer Reactions

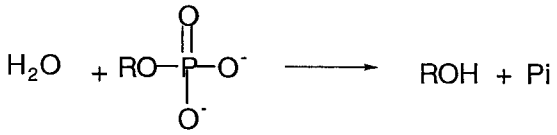
References:
Protein Catalysis
1. "Mechanism of Phosphoryl Transfer reactions, (1995) FASEB J. 1585-94, W. W. Cleland.
2. Isotope Effects to investigate phosphoryl transfer reactions, (2002) Accounts of Chemical Research 35, 105-112. A. Henge.
3. An overview of stereochemical questions (1992) The Enzymes 20, 95, 3rd edition, J. A. Gerlt.
4. "Why God Chose Phosphates" (1987) Science 235, 1173, F. Westheimer.
5. ATP hydrolysis (1995), Chemistry and Biology 2, 792, D. Hershlag.
6. Kinases and phosphatases (2001), Chemical Reviews 101, the entire august issue is devoted to this topic.
7. Trapping metaphosphate crystallographically (2003), J. Biol. Chem. 278, 16015-20, Chang, Fromm.
8. Trapping a pentacovalent phosphorous crystallographically (2003), Science 299, 2067, Dunaway-Mariano, K. Allen.

RNA catalysis:
1. Chemistry and Biology (1998) 10, 539-53, T. Cech.
2. Nature 395, 223-5 (1998), Bartel.
3. The structure of the ribosome at atomic resolution by Steitz, T. and Moore, P. reveals that peptide bond formation is catalyzed by RNA and not proteins! Many outstanding reviews have been written on this topic in the past few years.

Protein tyrosine phosphatases and Dual Specific phosphatases:
1. Reviews: J. Biol. Chem. 269, 31323-26 (1994).
2. Current Opinions in Chemical Biology (1998), 5, 633-641.
Papers that are the basis for the discussion in class:
3. J. Biol. Chem. 270, 379-803; Biochemistry 34, 3396-403 (1995); PNAS 93, 2493-2498 (1996); JACS 124, 10225-35 (2002).

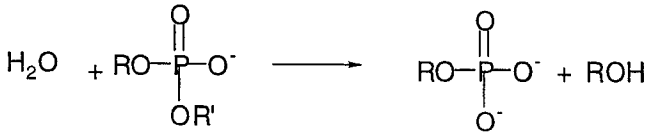
Overview: Scope of Reactions
1. Energy metabolism. ATP is the energy currency of the cell. ATP is kinetically stable and thermodynamically labile.
2. Signal transduction. Regulation via phosphorylation of proteins by kinases and dephosphorylation of proteins by phosphatases. Both GTP and ATP are used in these reactions.
3. Phosphates are found in nucleic acids and in many cofactors and vitamins (PLP, NAD, FAD, CoA etc).
4. RNA catalysis: The recent structure of the ribosome, RNA catalyzed formation of peptide bonds, has made RNA catalysts and their role in evolution, an active area of investigation.

Types of Reactions:
Phosphatases: These proteins can hydrolyze mono, di and tri esters of phosphates.



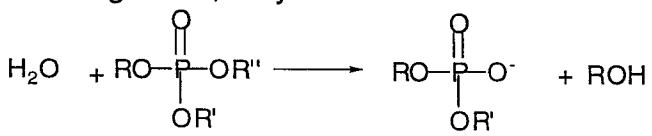
There are S/T phosphatases, Y phosphatases and dual specific phosphatases that can hydrolyze both S/T and Y's that are phosphorylated in proteins. There are also alkaline and acid phosphatases that work on small molecules, rather than proteins.

Phosphodiesterases:

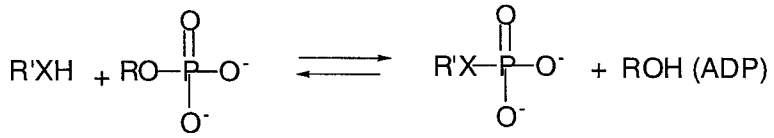


Dnases, Rnases (both exo and endonucleases), lipases, restriction enzymes. Most of these enzymes require metals for catalysis: Mg^{2+}, Zn^{2+}, Ca^{2+}.

Phosphotriesterase:
No naturally occurring PTEs, they have evolved to deal with pesticides.



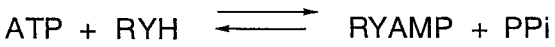
Kinases



R = ADP

(almost any amino acid side chain that is nucleophilic can be phosphorylated: S/T, H, Y, D, C. Many small molecules are also phosphorylated.

Nucleotidyl Transferase:



We will look at a few examples of phosphoryl transfer, before focusing our detailed studies on Y and Dual specific phosphatases. Reversible phosphorylation involves kinases and phosphatases and is an essential regulatory mechanism in all organisms. Phosphorylation can block an active site, can serve as an allosteric regulatory switch, can play a key role in protein-protein interactions.. Examples of reversible phosphorylation are glycogen biosynthesis and degradation, fatty acid degradation, pyruvate dehydrogenase (link between the glycolysis pathway and the Krebs cycle), cell cycle regulation, trans-membrane signaling

Examples we will briefly look at are Protein phosphatases, Rnase, Protein Kinase A.

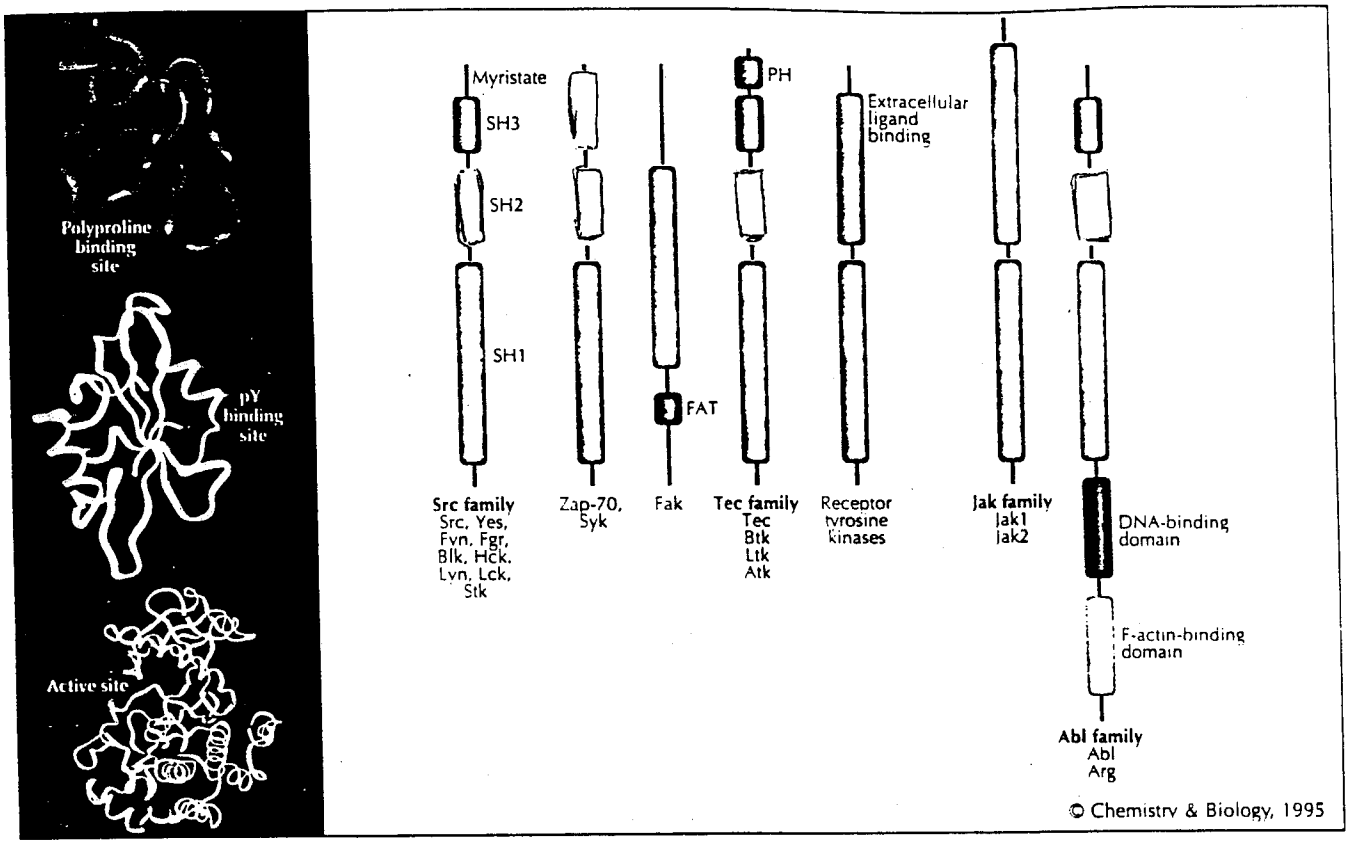


Fig. 2. Tyrosine kinases are made up of modular domains. Left: ribbon diagrams of the structures of SH3 [11] (top), SH2 [15] (middle) and SH1 [22] (bottom) domains (not to scale). Right: schematic representations of the domain structure of tyrosine kinases. Cytoplasmic tyrosine kinases contain at least one domain that mediates protein–protein association, such as the SH2 domain (color-coded yellow), SH3 domain (blue), pleckstrin homology domain (PH) or the focal adhesion targeting (FAT) domain of Fak. Src family kinases also carry a site for myristoylation, allowing their incorporation into the inner leaflet of the membrane bilayer. Abl also has a DNA-binding domain. The receptor tyrosine kinases, such as the EGF receptor, or the PDGF receptor, carry a domain for extracellular ligand binding. Hormone binding to this domain generally crosslinks the receptor molecules, causing the internal SH1 domains to associate and phosphorylate each other.

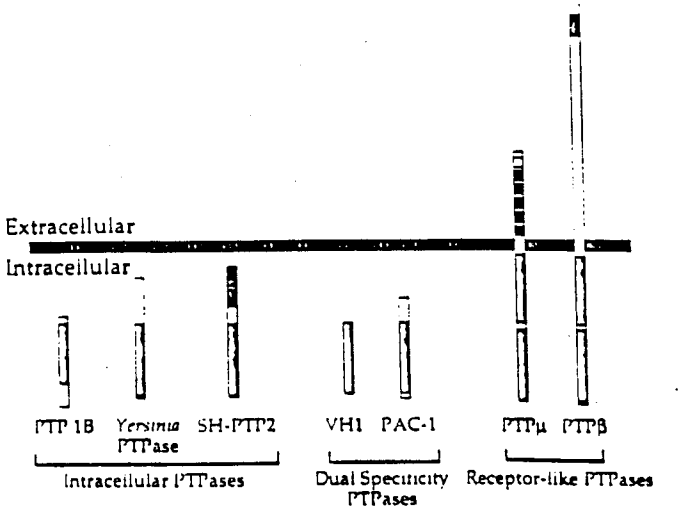
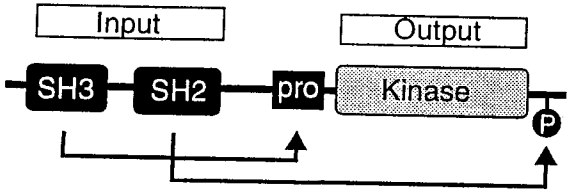


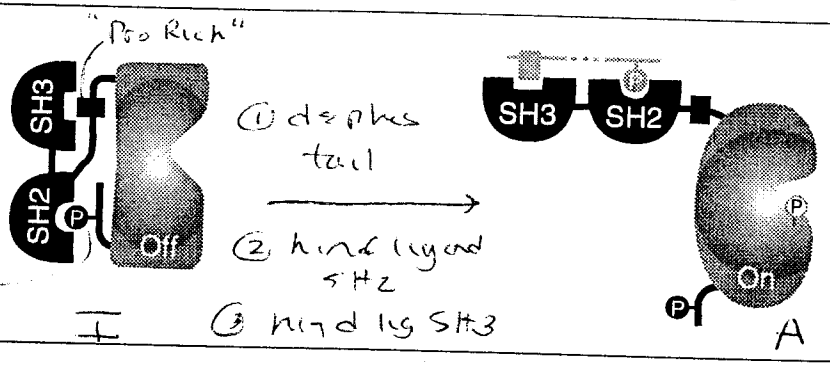
Fig. 3. **Diagrammatic representation of selected PTPases.** Members of the receptor-like (PTPμ and PTPβ), intracellular (PTP 1B, *Yersinia* PTPase, and SH-PTP2), and dual specificity (VH1 and PAC-1) classes are depicted. Functional motifs are illustrated in color: *red*, PTPase catalytic core; *green*, fibronectin type III repeats; *blue*, immunoglobulin-like domain; *yellow*, cysteine-rich motif; *gray*, carbonic anhydrase-like domain; and *magenta*, Src homology 2 (SH2) domain. The extracellular motifs of the receptor-like class are thought to mediate homophilic binding. The amino-terminal extensions of the intracellular PTPases may mediate intracellular localization.

both inhibit synergistically

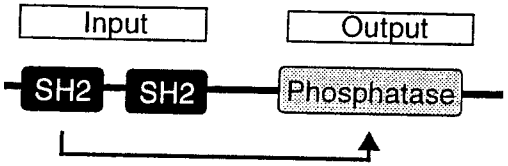
Domain architecture | Repressed state | Allosteric | Activated state

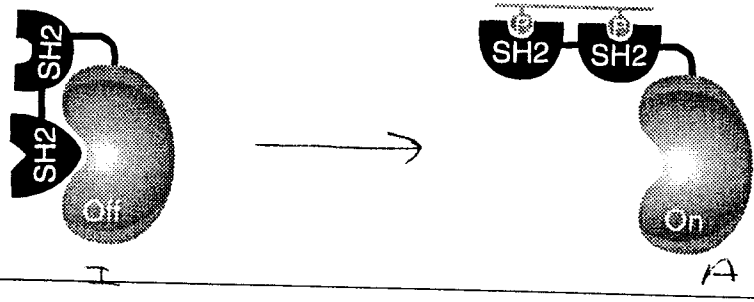
(a) Src Family (Y kinases)



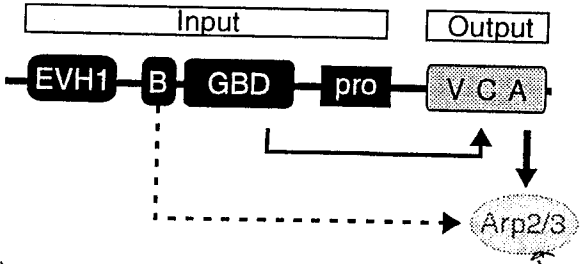


(b) SH-PTP2 phosphatase (tyrosine)



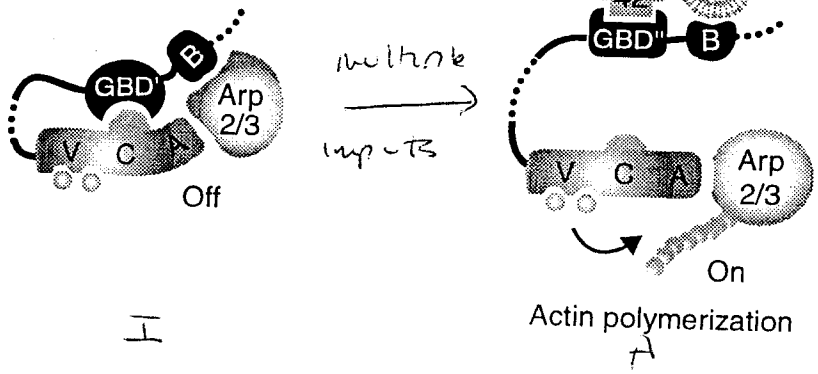


(c) N-Wasp - regulates Actin polymers

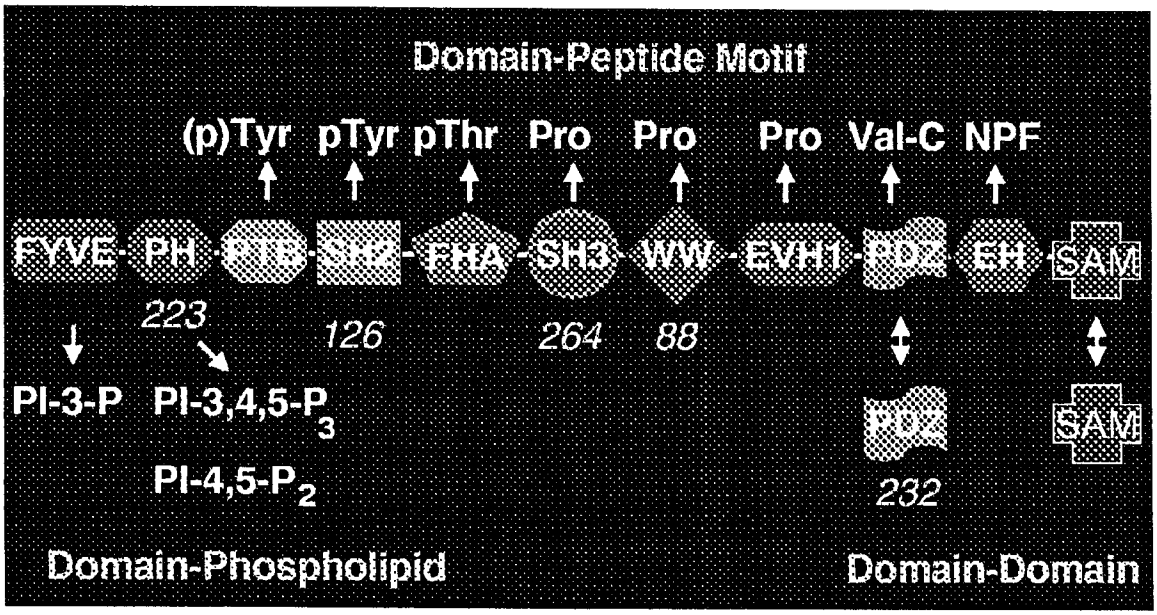


B = basic

7 proteins



G = G protein binding

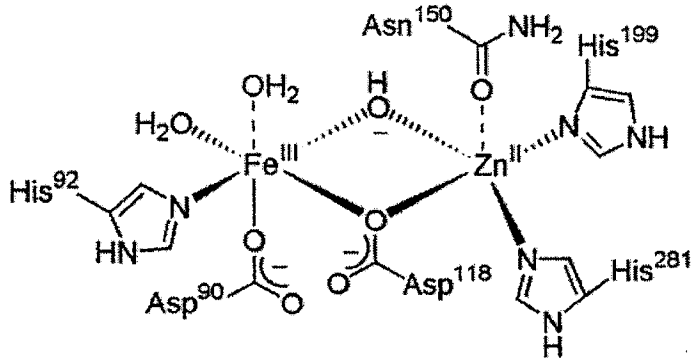


Modular domains of signaling proteins: interactive functions and numbers encoded in the human genome (PH, SH2, SH3, WW, PDZ

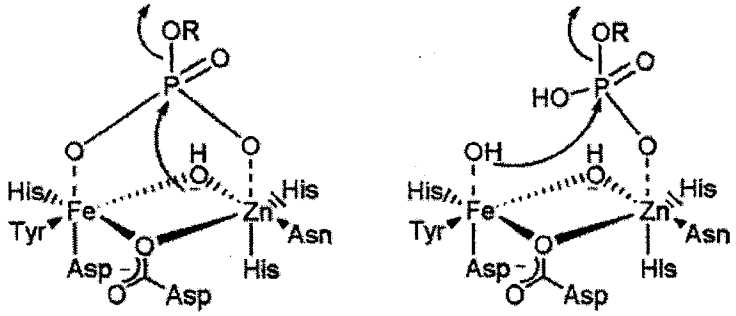
SH2 - recognize specific phospho-Y motifs

SH3 - recognize specific proline rich motifs

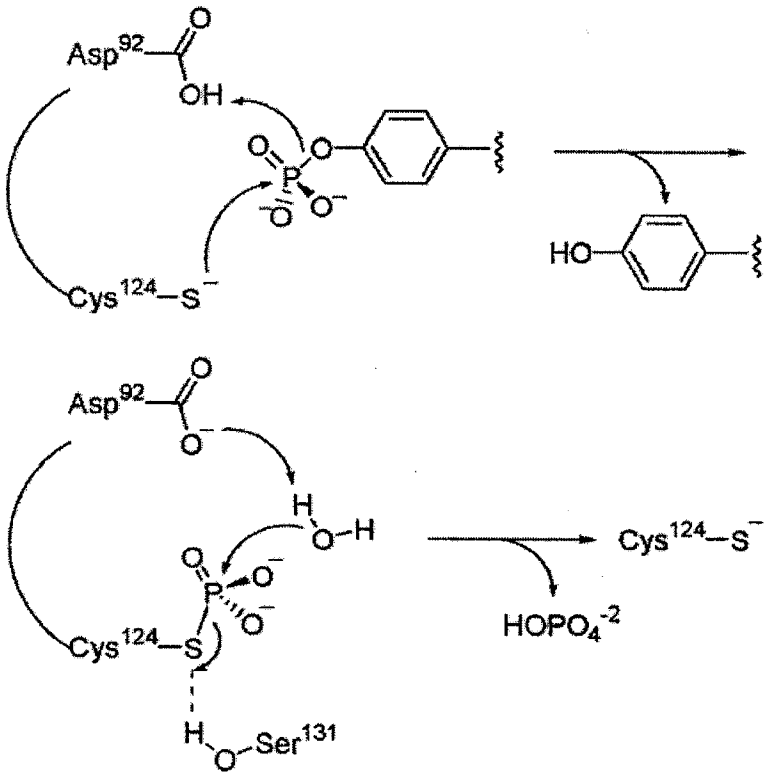
Serine/threonine specific protein phosphatases are the second major class of protein phosphates that catalyze dephosphorylation of proteins that contain phosphorylated amino acids. There are two distinct classes: the PPP gene family and the PPM gene familiy. The PPP family consists of a common catalytic subunit or domain that is associated with regulatory or targeting subunits or domains. The catalytic domain of the PP1 subunit can be associated with 15 regulatory subunits. The catalytic domain contains the conserved active site motif $DXH(X)_{23-26}GDCCDR(X)_{20-26}GNH(E/D)$.



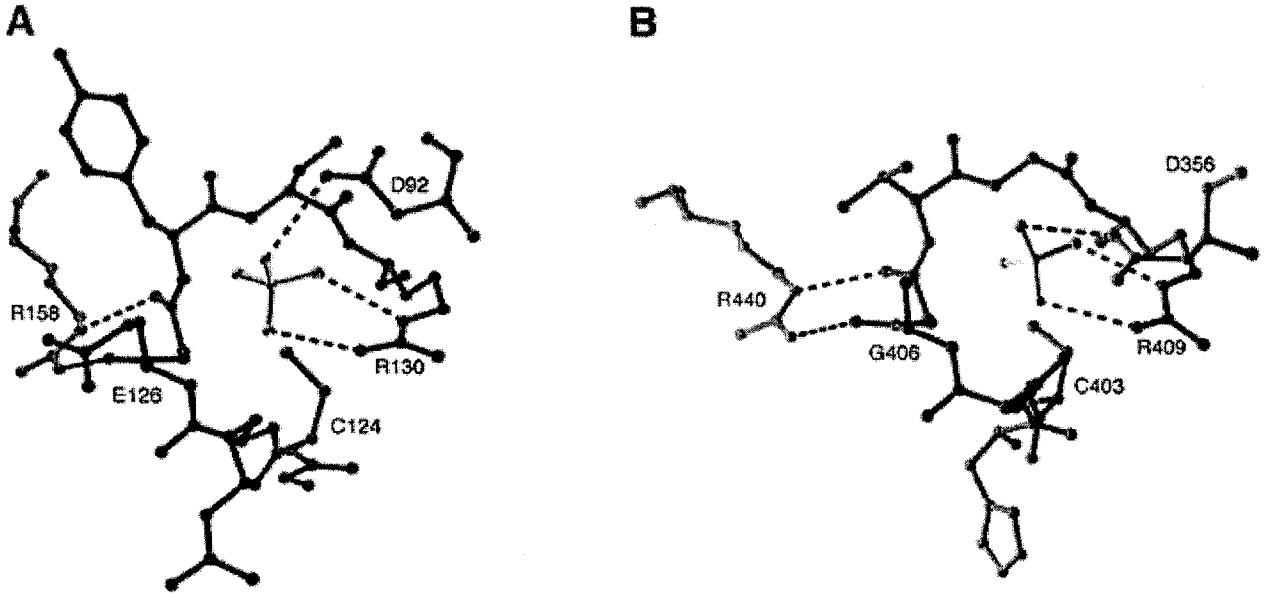
The postulated mechanism for this class of phosphatase is shown below:



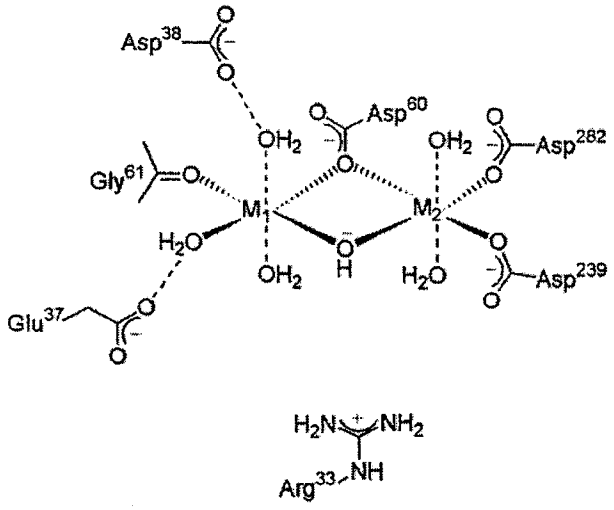
The PPM gene family contain 11 conserved motifs distributed along the full length of the protein.



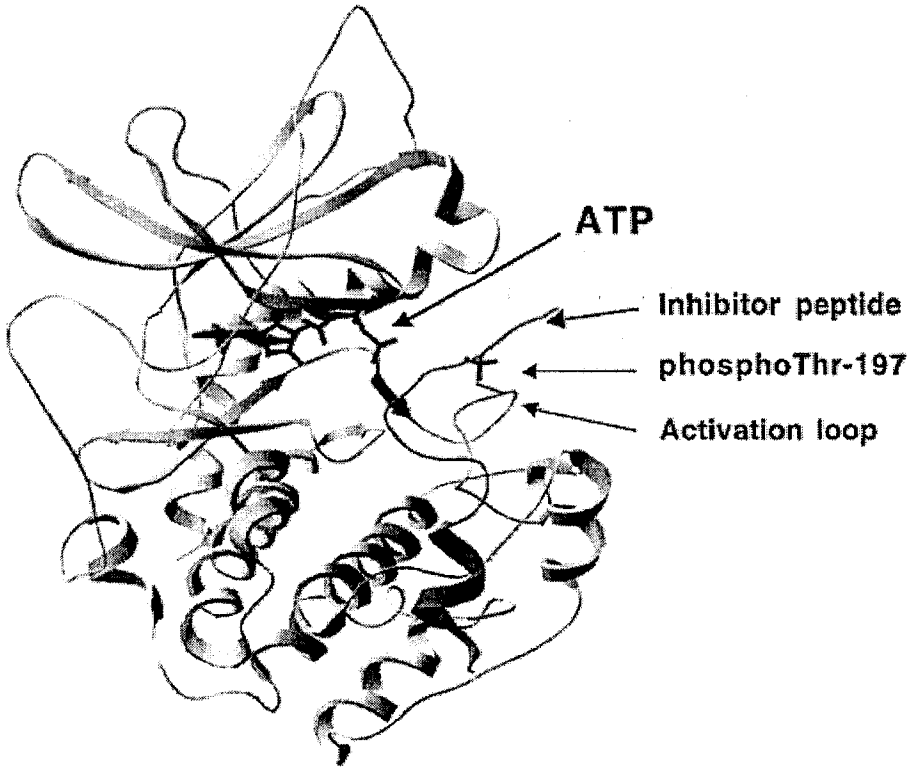
Proposed mechanism for PTPs (protein tyrosine phosphatases using VHR numbering of the important residues involved in catalysis.



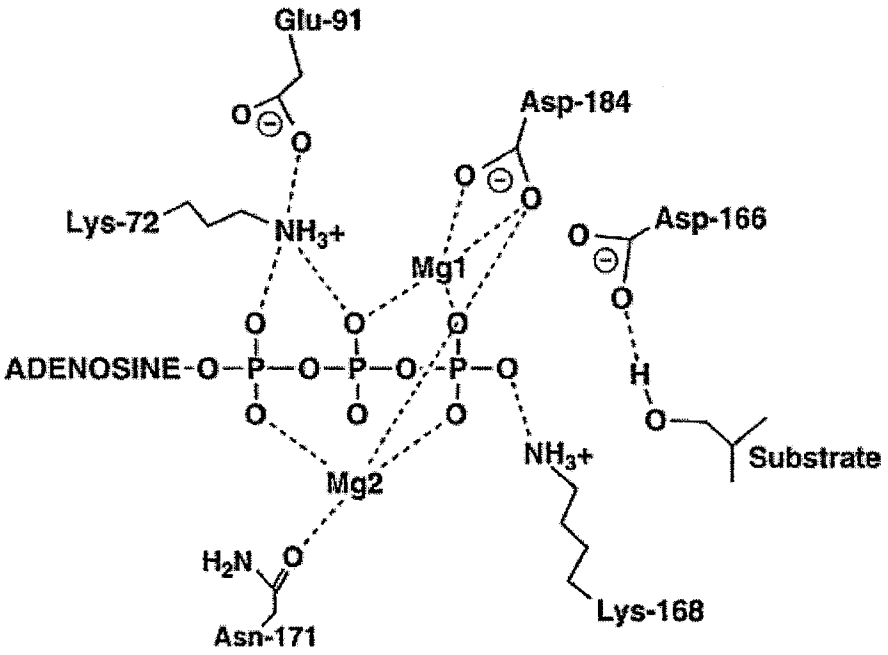
Three dimensional structure of the signature motifs of A. VHR and B. Yersinia PTP illustrating the conserved architecture of the active sites utilized by the PTPs.



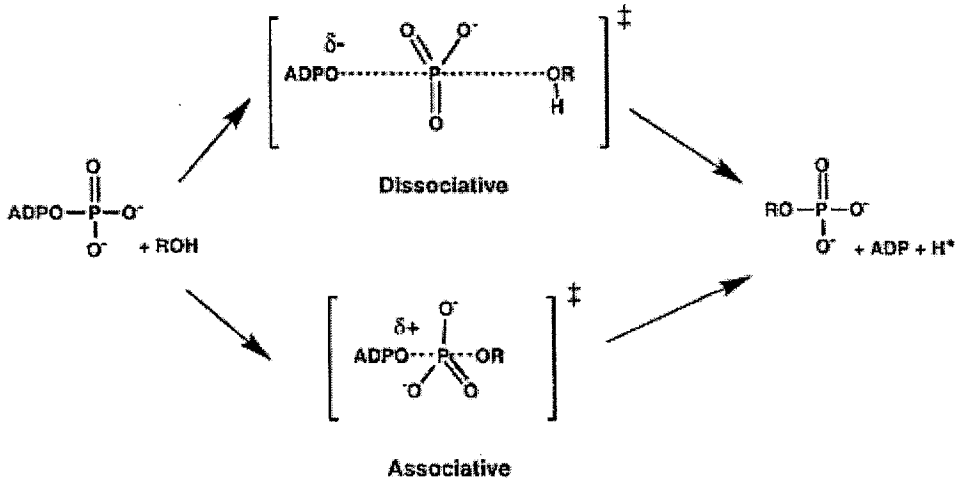
In the same issue of Chemical Reviews there is an excellent review on Protein Kinases 2001, 101, 2271-2290. These enzymes use ATP to phosphorylate the hydroxyls of serines, threonines or tyrosines of a variety of protein substrates. There are proposed to be 2000 of these types of proteins in the human genome. The core structure of these kinases has been conserved. These proteins, as with the phosphatases interact with a wide range of other proteins that regulate their activities. Finding the partners is a major focus of many laboratories.



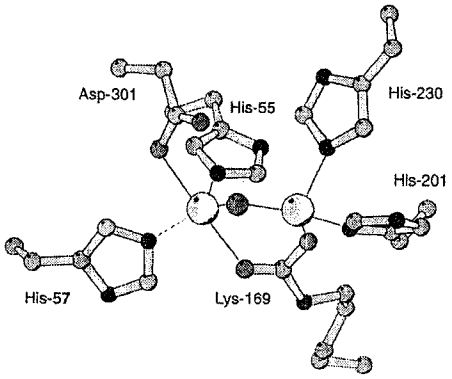
Key residues in the active site of the best studied of these kinases, protein kinase A (PKA) is shown below.



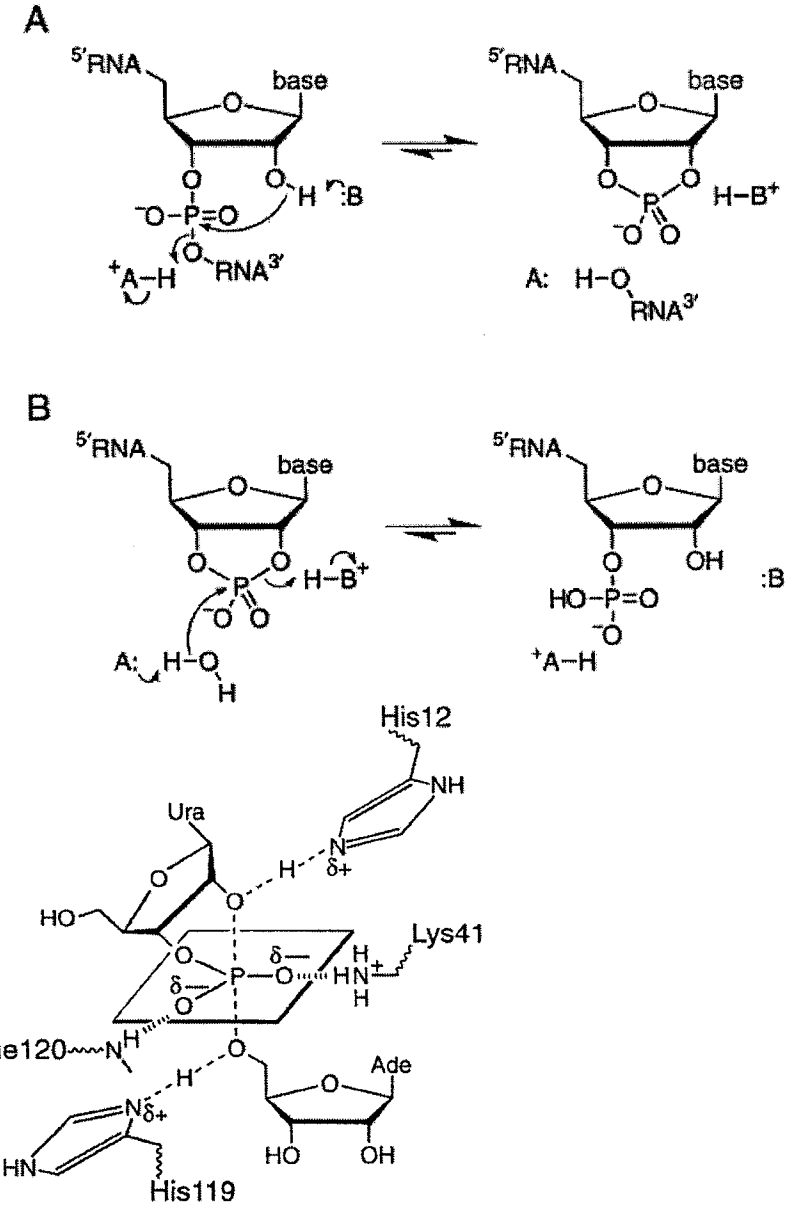
The mechanisms considered for phosphoryl transfer are either associative dissociative and are shown below. Evidence from a variety of sources favors a dissociative transition state for the kinases and the phosphatases.



Active site of phosphotriesterase:



Active site of Phosphodiesterases such as ribonuclease: (Chemical Reviews 98, 1045-66.



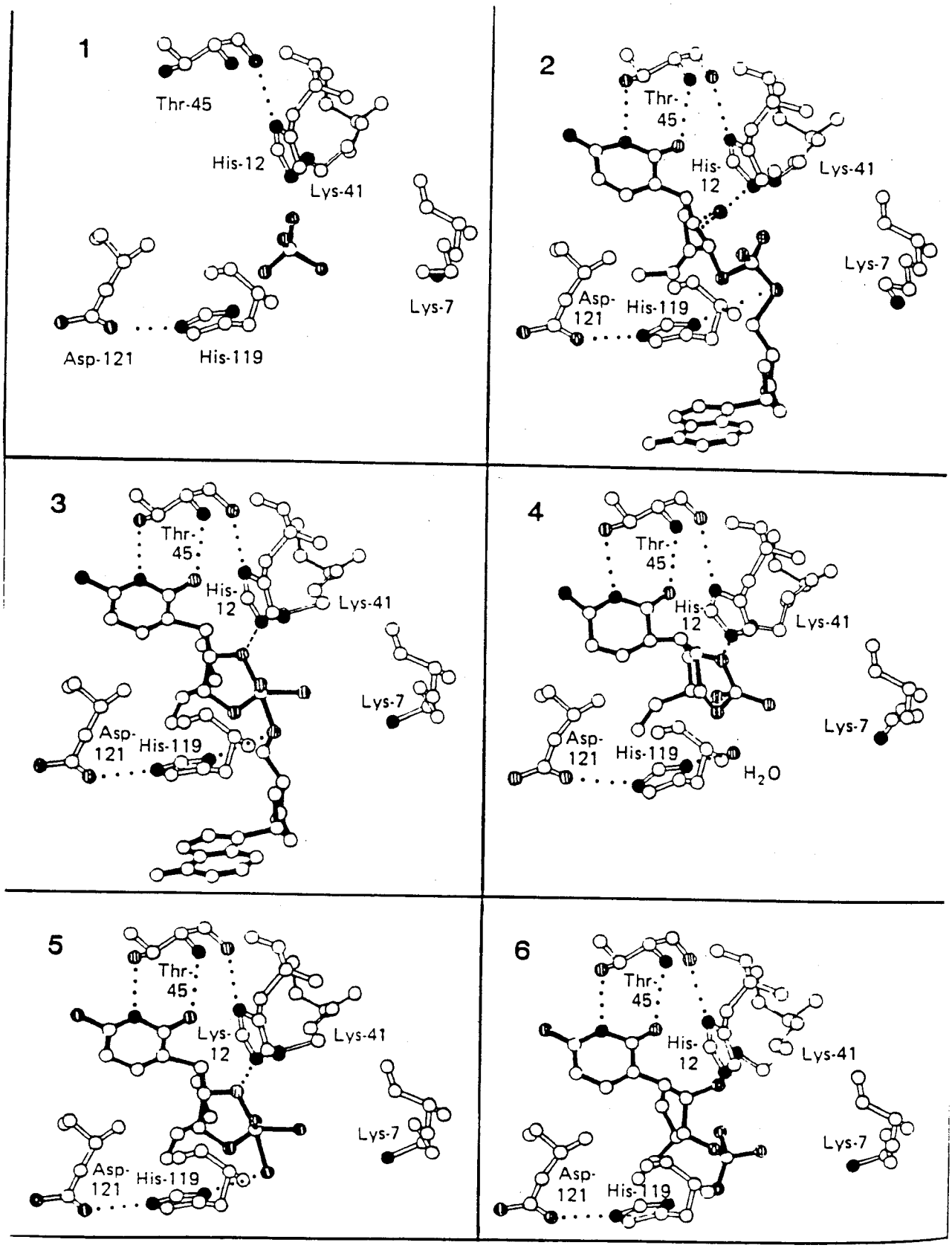


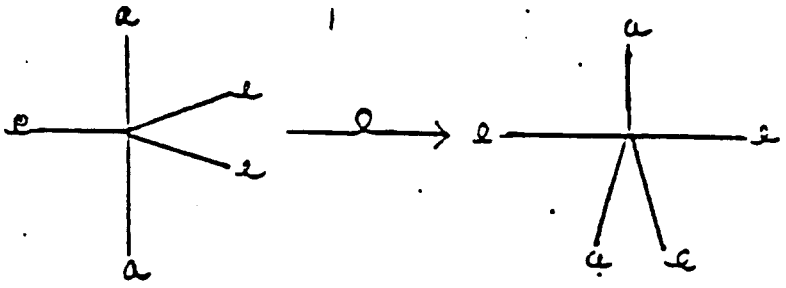
FIGURE 15-8. The structures of the ribonuclease active sites (frame 1) and the complexes with a substrate (frame 2), transition states (frames 3 and 5), the intermediate (frame 4), and the product (frame 6) (see text). [Modified from W. A. Gilbert, A. L. Fink, and G. A. Petsko (in press).]

Handout

PSEUDOROTATION AND RIBONUCLEASE

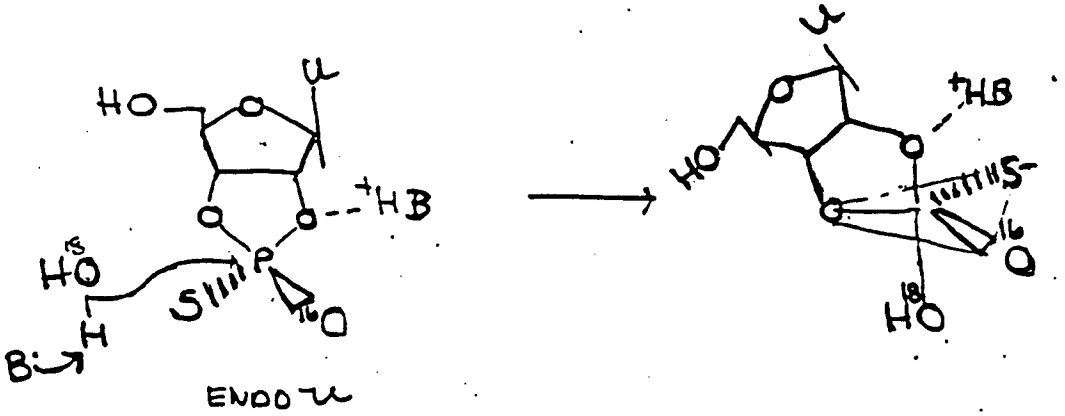
Pseudorotation Rules

1. Strain Rule ≡ 5 membered rings always span apical equatorial positions.
2. Polarity Rule ≡ electronegative ligands prefer apical positions (OR) whereas electropositive ligands prefer equatorial positions (O-, S-, SR).
3. Microscope Reversibility ≡ ligands enter and leave only from apical positions.

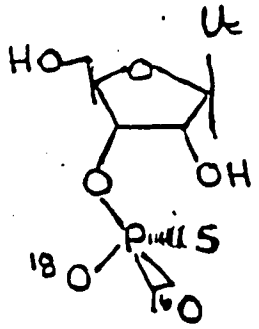


Use of Chiral Thiophosphates with Ribonuclease (RNase)

1. Inline $H_2{}^{18}O$ attack

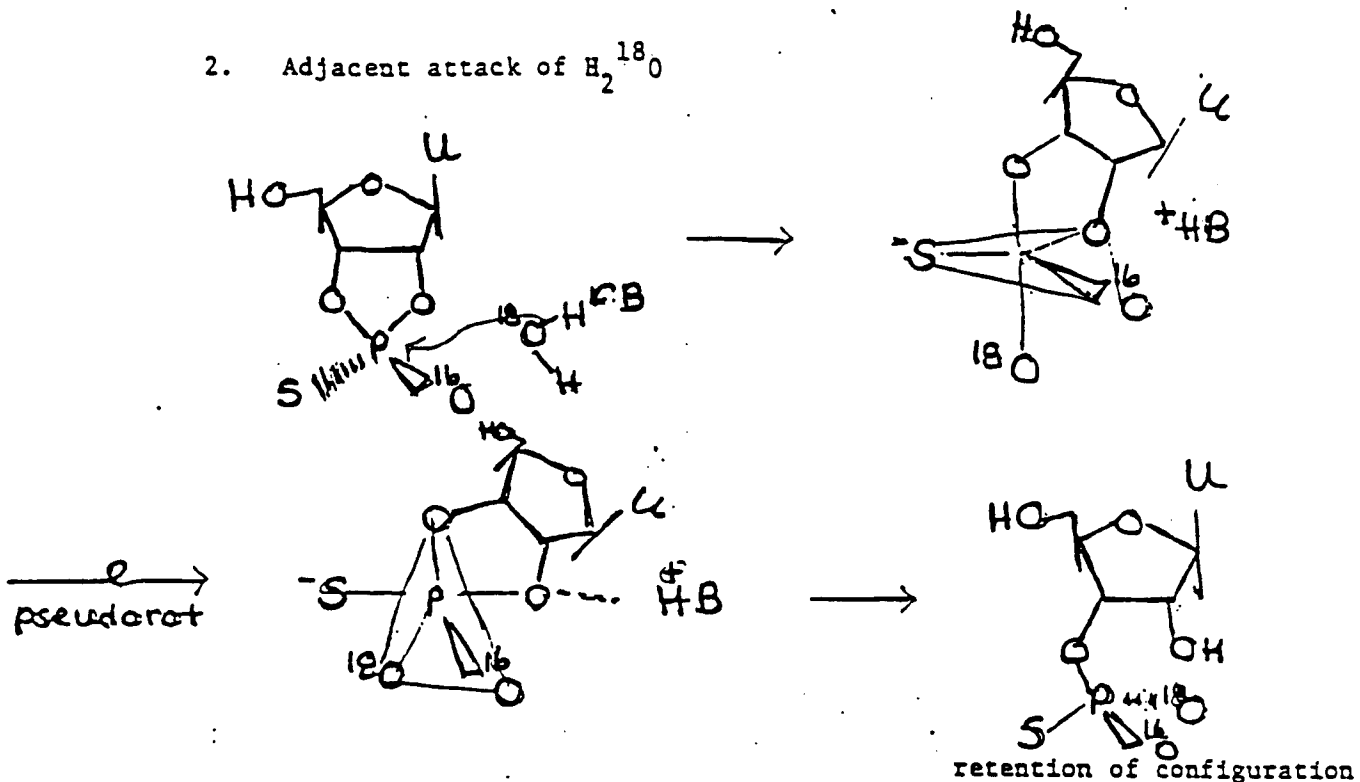


no pseudo rotation



Inversion of configuration relative to starting material

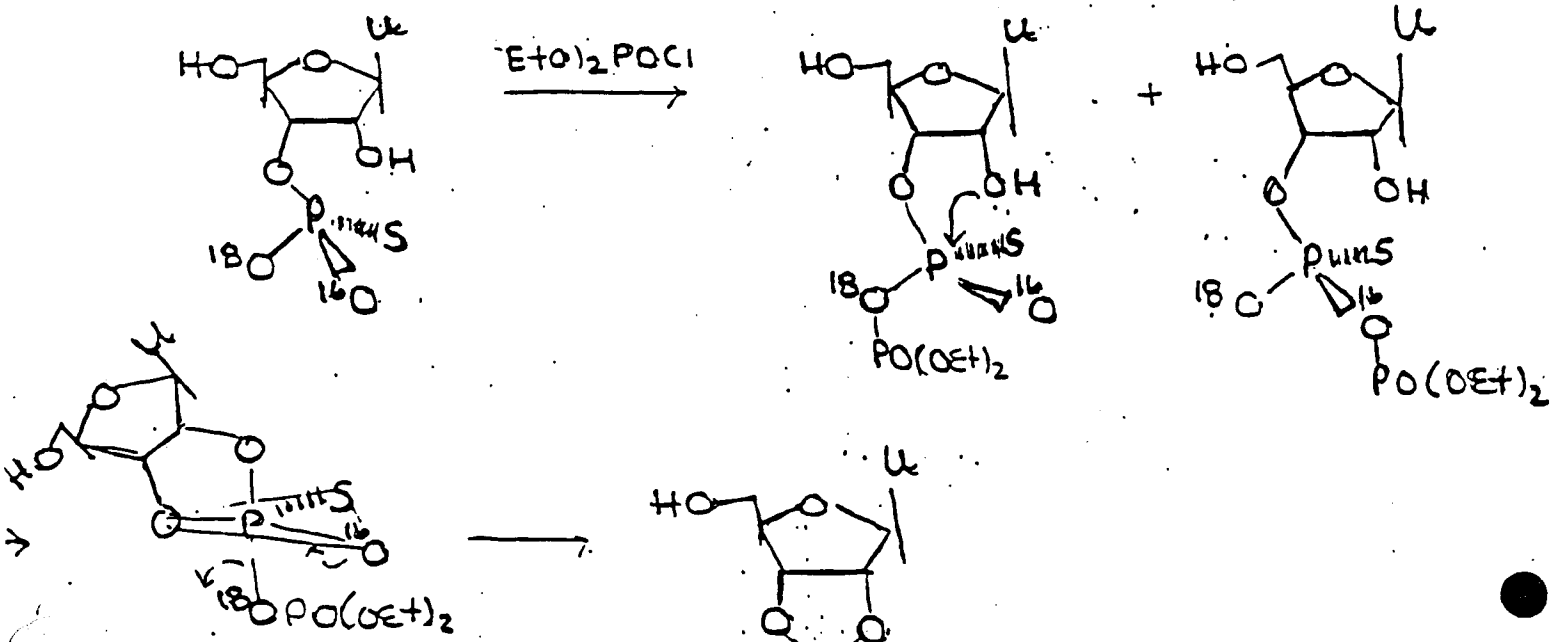
2. Adjacent attack of $H_2{}^{18}O$



relative to the starting material

Determination of the Configuration of 3'-UMP(S)

Using chemical reactions of known stereochemistry



No pseudo

that is starting material contains No ^{18}O

endo U > p(S) contains NO ^{18}O

and exo U > p(S) contains ^{18}O

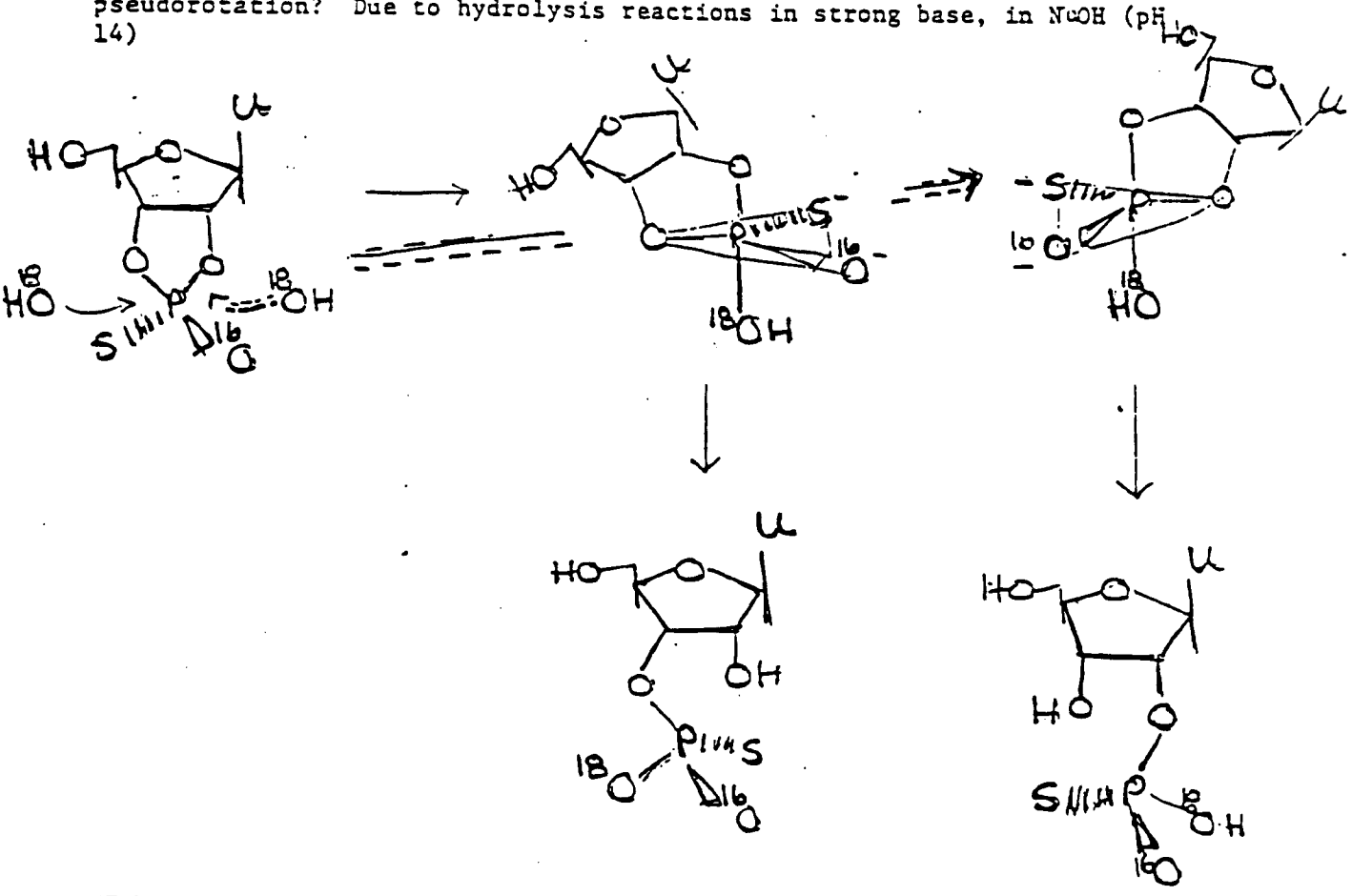
If one goes through the same reasoning for 3'-UMP(S) obtained from ring opening with retention, the results would be the opposite.

endo U > p(S) contains ^{18}O

exo U > p(S) contains ^{16}O

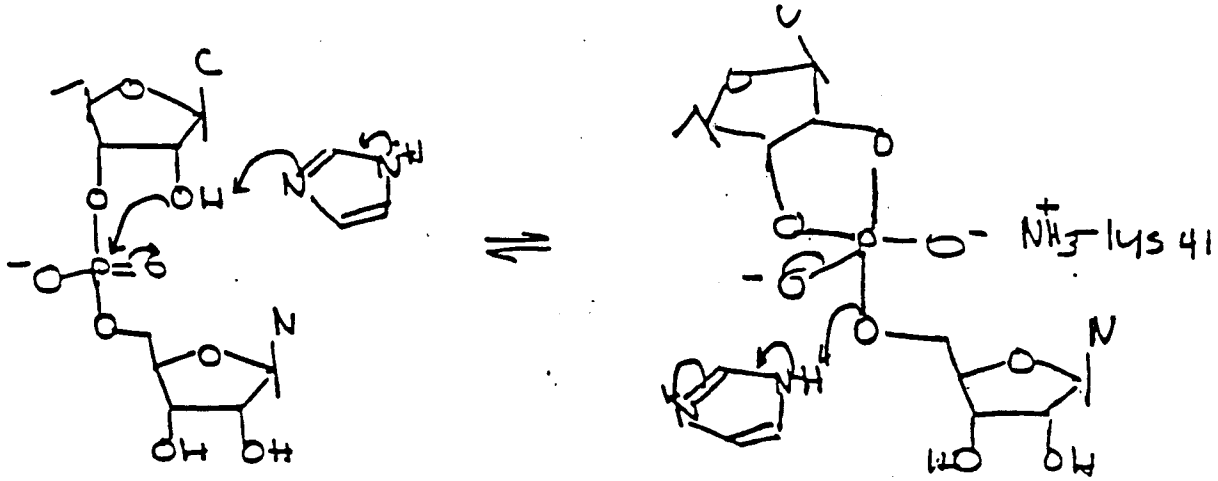
Problem: convince yourself this is true.

How does one know that this chemical ring closure proceeds without pseudorotation? Due to hydrolysis reactions in strong base, in NaOH (pH 14)

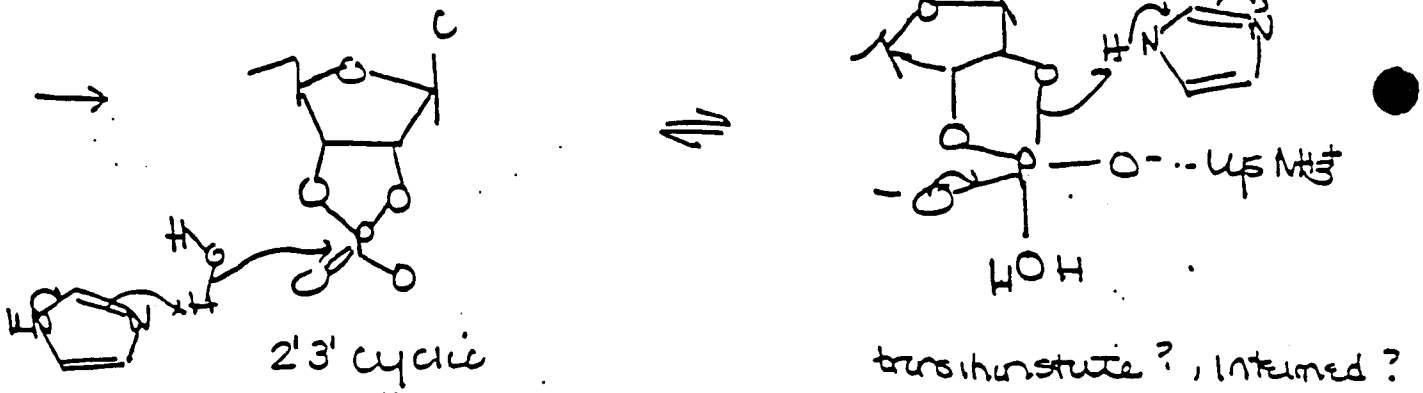


This presence of electropositive substituents in the equat. plane of above intermediates inhibit pseudorotation (planarity Rule). Therefore the ring opening is an inversion.

Proposed Mechanism for Ribonuclease

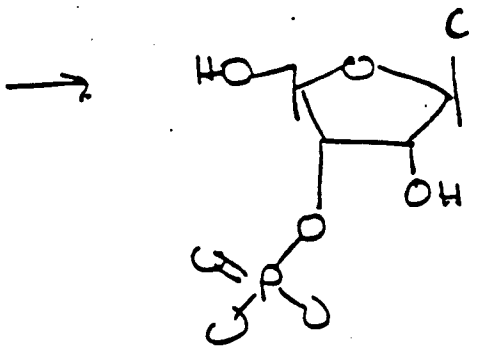


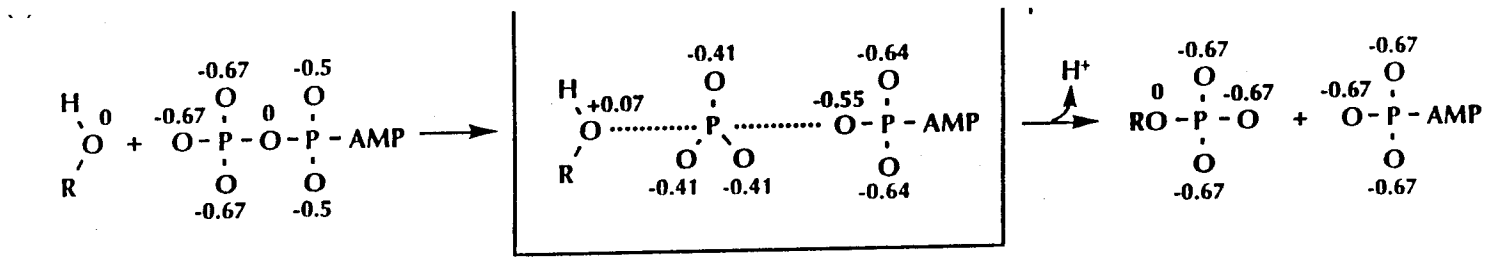
transition state?, intermediate?



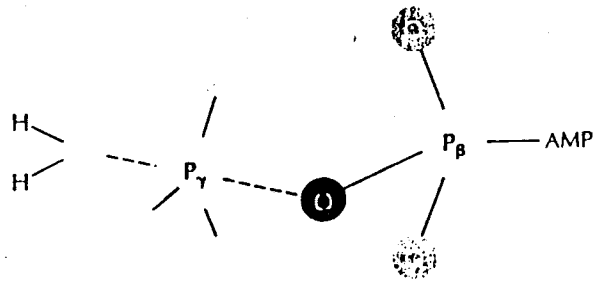
2'3' cyclic phosphate.

transition state?, intermed?



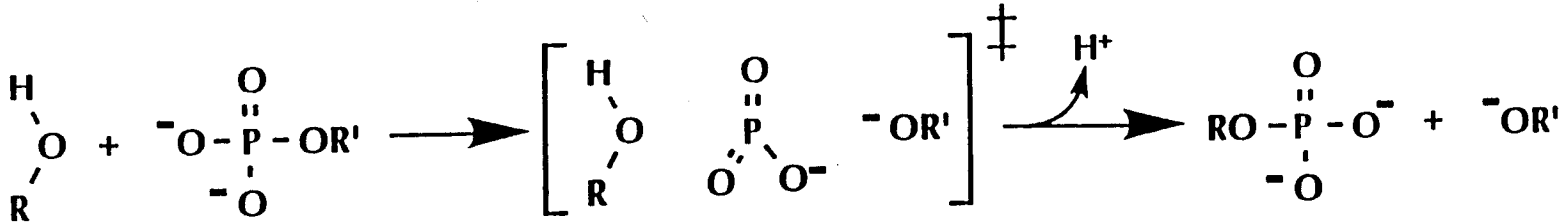


(b)

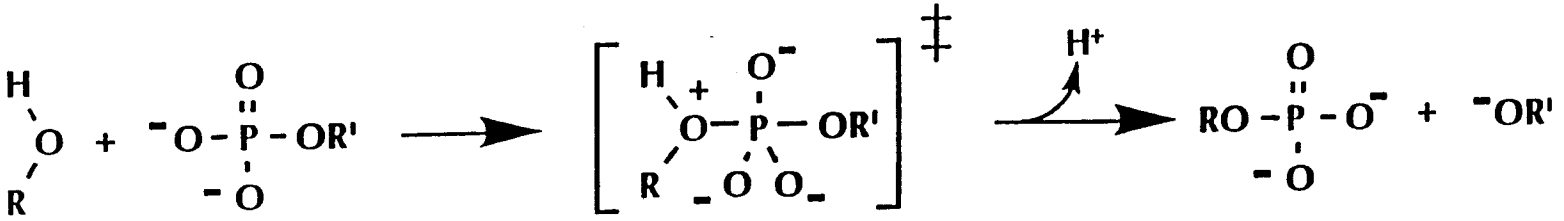


Transition state for ATP hydrolysis Admiraal and Herschla

(a)



(b)

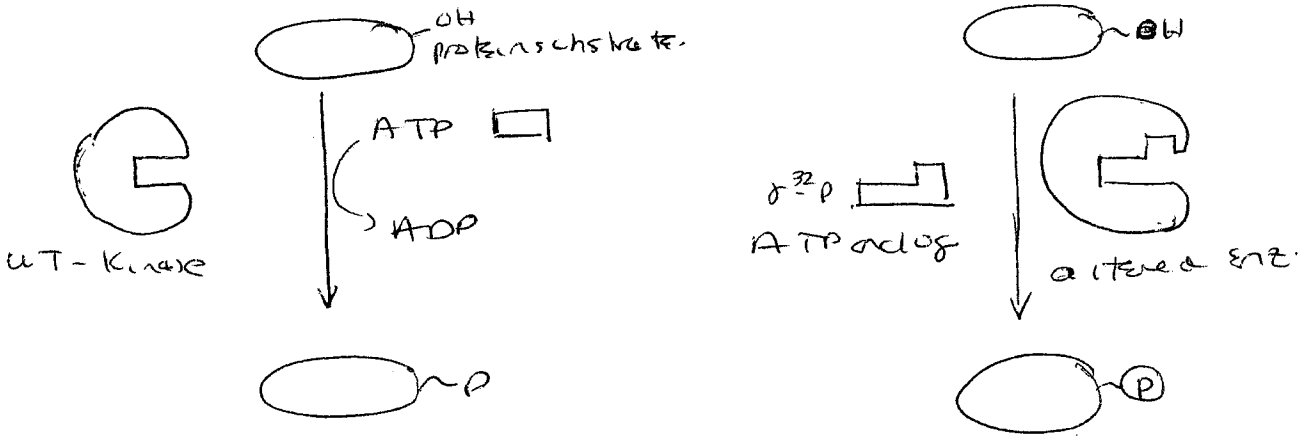


Specificity of Kinases:
Use of Phage display, combinatorial libraries, antibodies that specifically recognize phosphorylated amino acids, a review: Sci STKE 2002 162, PE49, L. C. Cantley

Use of modified ATP analogs and modified kinases: Shokat K.
Methods in Mol. Biology 2003, 233, 253-271; Current Opinion in Chemical Biology 2001, 360-7.

As with proteases, understanding the substrates for kinases (and phosphatases) is a very difficult problem, both in vitro and in vivo. A clever method using genetic engineering, structure and chemistry has been developed, although it is not without its problems. One has a library of ATP analogs. The kinase of interest (whose substrate that you wish to determine) is then engineered using structure and mutagenesis methods to bind the ATP analog and not the ATP itself (or with greatly reduced activity and affinity). The ATP analog and mutated kinase (an orthologous pair) then can be used in vivo in an effort to find the kinase's actual substrate. One must use and organism in which the wt-kinase can be replaced with the mutant kinase. One then uses [γ^{32}P]-ATP analog and asks what proteins in the cell are modified by this mutated ATP?

Problems: 1. The ATPs, because of phosphorylation will not readily get into cells. One would like to carry out these experiments under different growth conditions, but the membrane barrier may require that these types of experiments be carried out in crude cell extracts.
2. Need to carry out these experiments in an organism (yeast) where it is easy to replace the wt-gene with the mutant gene.
3. The activity and stability of the engineered protein must not be compromised.



Data for Dual-Specific Phosphatases

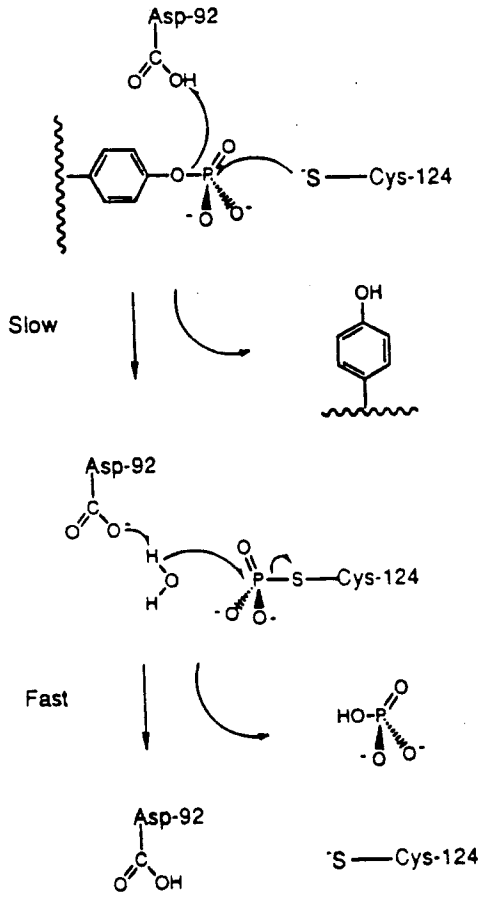


FIGURE 6: Proposed catalytic mechanism for the hydrolysis of phosphate monoesters by the dual-specific phosphatases.

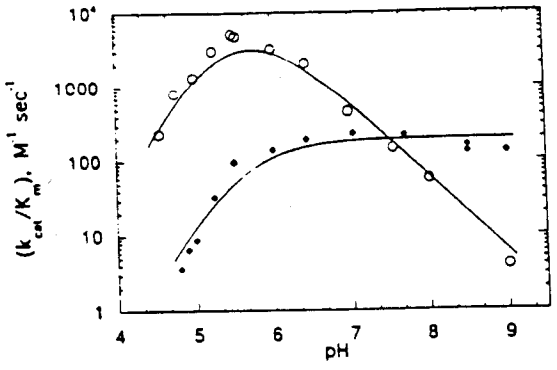


FIGURE 3: pH dependency of the k_{cat}/K_m value for native and D92N mutant VHR. The open circles represent the native enzyme data, and the solid diamonds represent the D92N mutant data. The native and D92N enzyme data were fitted to eqs 4 and 5, respectively. Results are given in Table 1. See Materials and Methods for details.

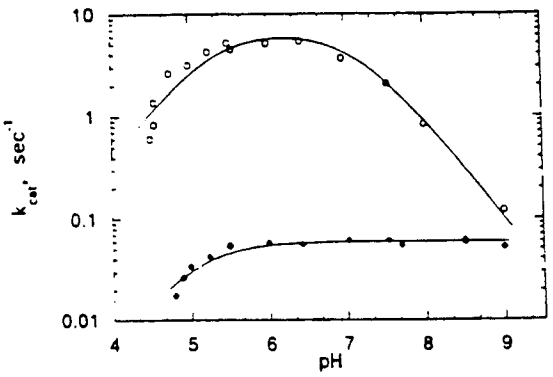


FIGURE 4: pH dependency of the k_{cat} value for native and D92N mutant VHR. The open circles represent the native enzyme data, and the solid diamonds represent the D92N mutant data. The native and D92N enzyme data were fitted to eqs 3 and 2, respectively. Results are given in Table 1. See Materials and Methods for details.

Table 2: Site-Directed Mutational Analysis of VHR[a]

enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
native VHR	5.14	1.59	3240
E6Q	5.92	2.29	2590
E32Q	4.83	1.73	2800
D110N	2.73	1.56	1755
D14N	4.85	2.65	1830
D92N	0.059	0.42	141

[a] Each data set was fitted to eq 1 as described in Materials and Methods. Standard errors were omitted for clarity. Average errors on the fitted parameters were approximately 5% and never more than 10%.

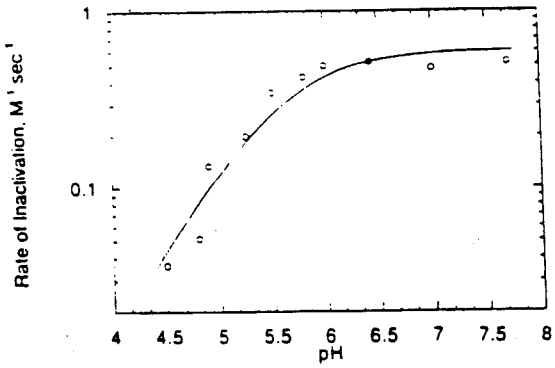
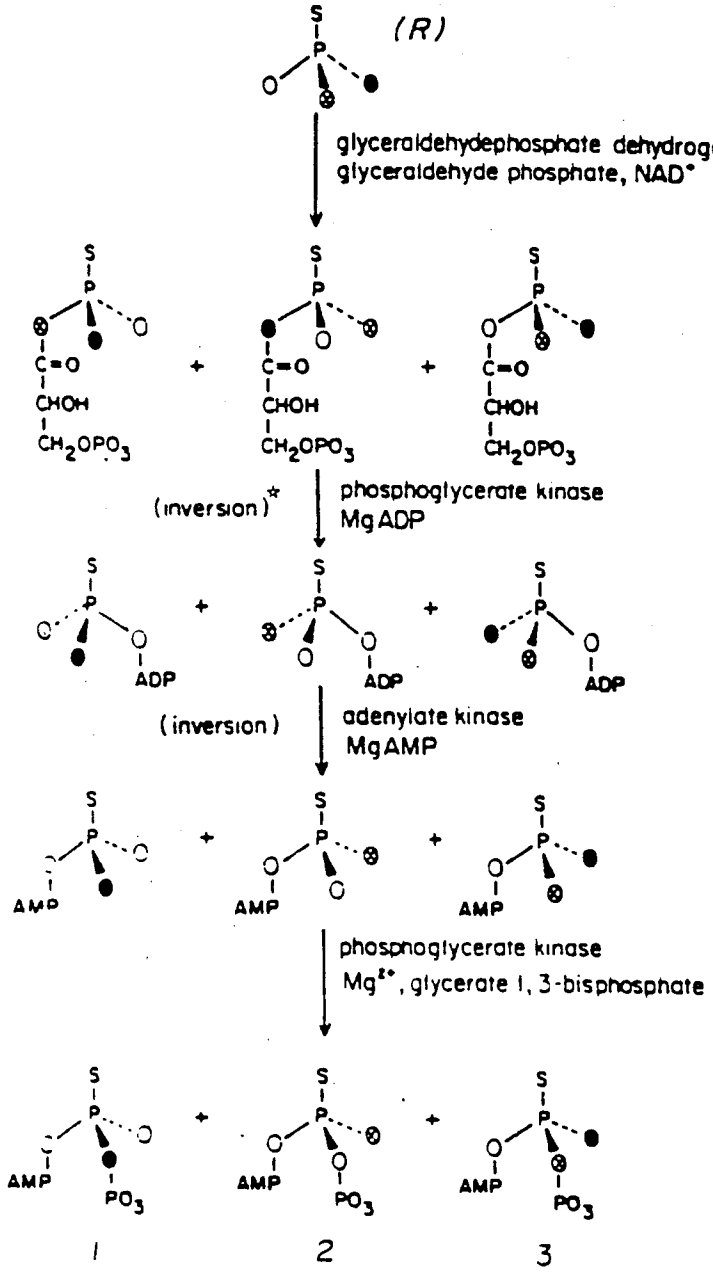


FIGURE 5: pH dependency of the rate of inactivation of VHR by iodoacetate. The data were fitted to eq 2. Results are given in Table 1. See Materials and Methods for details.



SCHEME 2. *The stereochemical course of this reaction was previously unknown. In this scheme, only the products due to inversion at the transferred phosphorus are shown, since the results show that this is the mechanism.

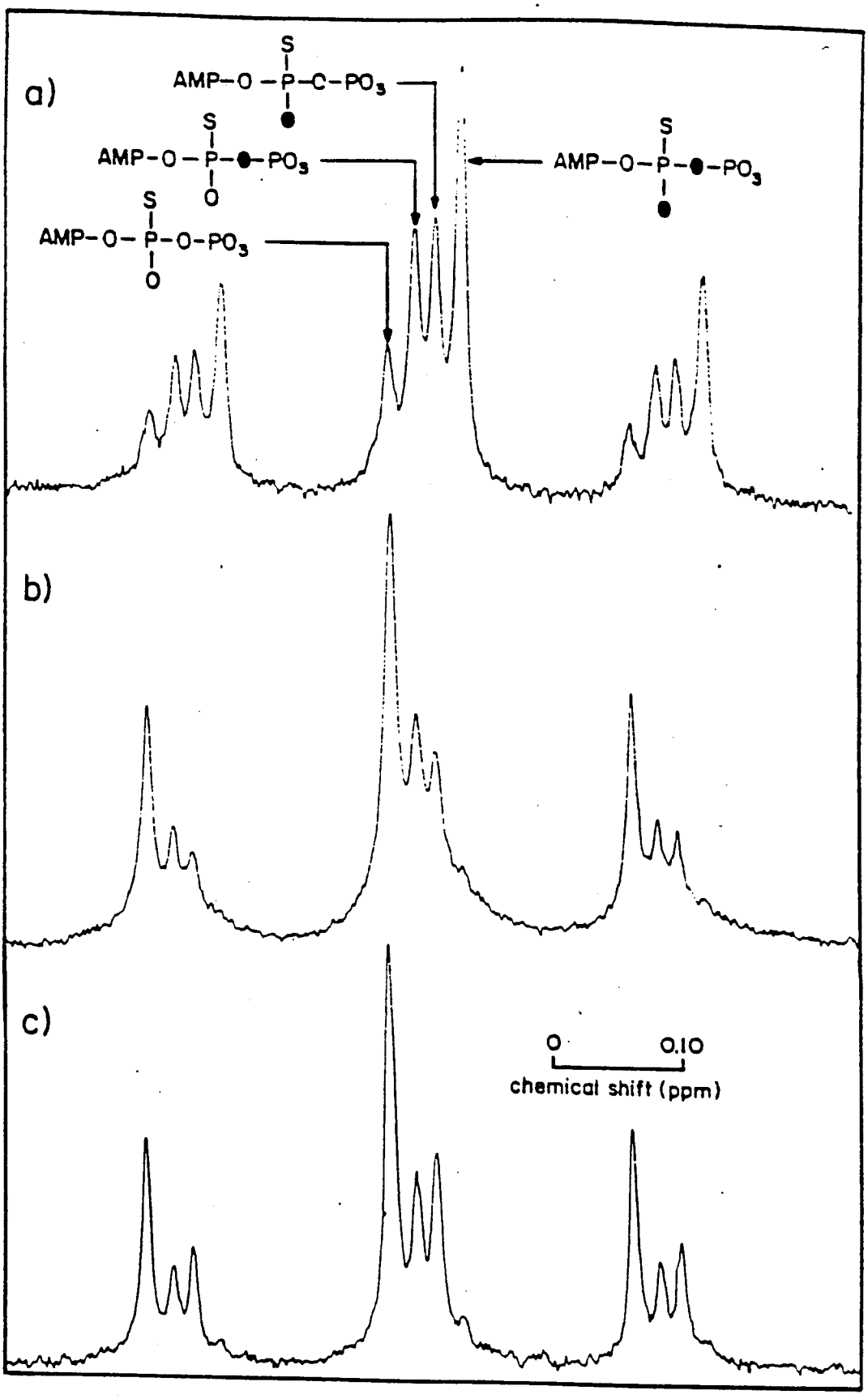
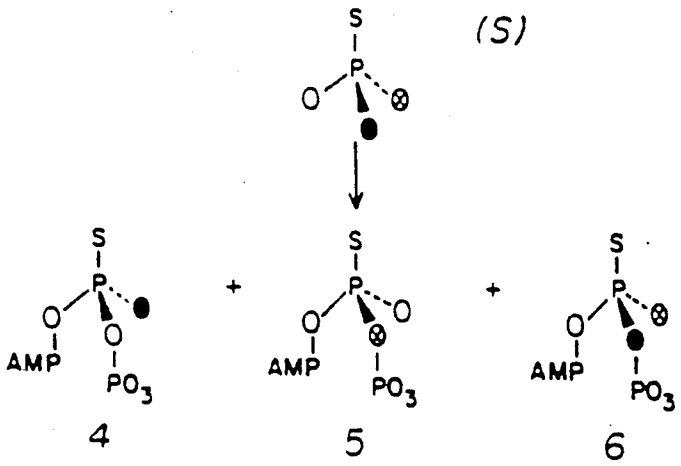


FIG. 1. **^{31}P NMR spectra.** The ^{31}P NMR spectra of the β-phosphorus of (*a*) [β-^{18}O; $\beta\gamma$-^{18}O]ATPβS, for which the isotopic enrichment is 70%; (*b*) ATPβS derived from the *R*-enantiomer of inorganic [^{16}O, ^{17}O, ^{18}O]thiophosphate, and (*c*) ATPβS derived from the *S*-enantiomer.



SCHEME 3