CHAPTER-1
INTRODUCTION

Phosphorylases are glycosyl transferring enzymes, specific for the glucose part of the molecule and the break comes next to the glucose carbon (1).

The typical phosphorylase reaction is

\[ A - G + P \rightleftharpoons A + G - P \]

where G represents a glycosyl group, P represents phosphate and A the other glycosyl acceptor. The specificity towards both A and G must be considered. Each phosphorylase is highly specific for one particular glycosyl group in the role of G. Thus α-Glucan phosphorylase (EC 2.4.1.1) is completely specific for α-D glycosyl transfer. In the case of maltose phosphorylase (EC 2.4.1.8) the glycosyl group undergoes an inversion during transfer, so that in one direction the enzyme is specific for α-D glycosyl and in other direction for β-D glycosyl residues.

A (other glycosyl acceptor) is also having a fairly high degree of specificity. Thus, for the starch phosphorylase of potato, A must be a polysaccharide chain of at least four glucose units, although very slight activity can be detected with three (2, 3, 4). The glycosyl group is transferred only to the 4-position of the non-reducing terminal unit. For good activity all the links in the chain must be 1,4-β-glucoside links; lichenin (containing 1,4-β-glucoside links) and dextran containing 1,6-β-glucoside links) are not acted upon (4) and glycogen is acted on much more slowly and less completely than by the animal enzyme (5). Branched dextrins derived from amylopectin and containing one or two 1,6-β-glucoside links instead of 1,4-β-glucoside links, may be actually inhibitory (3). Dextrins of four or more units
containing a phosphate group on the 6-position of the second or third glucose residue can act, but the presence of this phosphate group reduces the activity to one fifth (6).

The muscle glycogen phosphorylase differs significantly from the potato enzyme in that it does not act with the small straight chain dextrins and works best with larger branched molecules. In the phosphorolysis it acts much more rapidly on amylopectin or glycogen than on amylose, and in the reverse direction it can act with the limit dextrin produced from amylopectin or glycogen by the phosphorolysis, though not with the limit dextrin produced by β-amylase, which contains four fewer glucose residues in the main chain (7, 8). The specificity is somewhat influenced by the size of the substrate molecule, the liver enzyme working best with smaller glycogen molecules than the muscle enzyme (9).

Since glucose-1-P is a stronger acid than inorganic phosphate, the equilibrium constant is highly pH dependent. At pH 6.8, the Pi/glucose-1-P ratio is 3.6 (10). However, the enzyme functions in the direction of glycogen degradation in vivo because the ratio of Pi/glucose-1-P greatly exceeds the equilibrium constant determined in vitro (11, 12).

Glycogen phosphorylase assay can be done by following either the forward or the backward reaction. In the direction of glycogen degradation, the enzyme is assayed using a coupled enzyme assay system by which the liberated glucose-1-P is estimated (13). The activity is measured in the direction of glycogen synthesis using the substrate glucose-1-P and glycogen (14) and estimating the liberated inorganic phosphate colorimetrically. The convenient colorimetric method of Fiske and SubbaRow (15) for inorganic phosphate has been widely used.

Phosphorylase is the key enzyme in the mobilization of chemical energy from glycogen. It is a complex allosteric protein that is subject to activation and
inhibition by chemical stimuli, including those of two other enzymes: phosphorylase kinase (ATP : phosphorylase phosphotransferase, EC 2.7.1.38) which activates it by phosphorylation of one specific pair of Ser-14 residues and phosphorylase phosphatase (phosphorylase phospho-hydrolase, EC 3.1.3.17) which inhibits it by hydrolysis of the serine phosphate bonds. In muscle, phosphorylase kinase is activated normally by the same release of calcium ions from the sarcoplasmic reticulum that also stimulates contraction. When activated, phosphorylase catalyses the stepwise phosphorolysis of glycogen with release of glucose-1-phosphate. Under in vivo condition it is a dimer of two identical subunits, each containing a single polypeptide chain of 842 amino acid residues to which a pyridoxal phosphate is attached by a schiff base at lysine-680 (16).

Phosphorylase b is the unphosphorylated form. The molecular weight of the rabbit muscle phosphorylase monomer calculated from the amino acid sequence is 97,412 (17). This includes the N-terminal acetyl group and phosphoryl group at Ser-14. The subunit relationship of the phosphorylated a form of the enzyme depends on conditions like enzyme concentration, pH, temperature, ionic concentration etc. (18). At low enzyme concentrations, the rabbit phosphorylase a exist as a dimer (13). The phosphorylase a from lobster, (19, 20), crab (21) and sepia (22) exist exclusively as a dimer. The dimeric and tetrameric forms of phosphorylase a have been found in tissues of a number of other species like man, shark, rat and frog (18). In the mussel, Mytilus edulis it is shown that monomeric and dimeric forms of phosphorylase b exist in equilibrium (23). The molecular weight in all these cases has been found to be in the range of 90,000 to 100,000 for the monomer.

Phosphorylase b is inactive under in vivo condition, but can be activated in vitro, weakly by inosine monophosphate (IMP) and strongly by adenosine monophosphate (AMP), when it reaches 80 per cent of the activity of the phosphorylated
Regulation of phosphorylase a and b by effectors
form. The phosphorylated form, known as phosphorylase \( a \) exhibits nearly maximal activity without AMP. Each of the two forms is subject to regulation by effectors as given in Fig.1-1.

Phosphorylase \( b \) shows an absolute requirement of AMP for activity (24). In the absence of AMP the activity of rabbit muscle phosphorylase \( b \) is less than 1% of that in the presence of AMP. Phosphorylase \( a \), on the other hand, is active without AMP although it is about 20-40% activated by its presence depending on the concentration of the substrate (25).

\[
\text{The ratio of activity} \quad \frac{-\text{AMP}}{+\text{AMP}}
\]

for phosphorylase \( a \) can be as high as 80%.

The properties and interaction of phosphorylase, phosphatase and kinase have been studied in a glycogen complex isolated from rabbit muscle in which all the enzymes and glycogen are held together. Interconversion of phosphorylase \( a \) and \( b \) has been demonstrated in this complex (26). Addition of ATP, calcium and magnesium to a solution containing this complex has been shown to be accompanied by conversion of phosphorylase \( b \) to \( a \) which when all ATP is used up is reconverted to the \( b \) form (26, 27).

A complex of muscle phosphorylase \( a \) and alanine amino transferase has been purified, and it has been shown that metabolites which affect the activity of phosphorylase, such as AMP, glucose, glucose-1-phosphate and glycogen, also affect the activity of the transaminase in this complex (28).

Glycogen metabolism by mammalian tissues involves at least four enzymatically catalysed steps as given in Fig.1-2 (29). The initiating influence in the
Fig. 1-2

Steps in the regulation of mammalian glycogen phosphorylase

AC is adenylate cyclase (EC 4.6.1.1); R and C are the regulatory and catalytic subunits of protein kinase (EC 2.7.1.37); PhK is phosphorylase kinase (EC 2.7.1.38). GP is glycogen phosphorylase (EC 2.4.1.1). Other abbreviations are: cA = cyclic AMP; Gly = glycogen; G-1-P = glucose-1-phosphate. The enzyme-catalysed steps are numbered 1-4.
case is the activation of the membrane-bound adenylate cyclase (AC), caused, depending on the tissue concerned, by the binding of a hormone, to a specific receptor located in the same membrane. The cyclic AMP generated interacts with an inactive protein kinase, stimulates the dissociation of an inhibitory regulatory subunit (R) from it, leaving an active catalytic unit (C) which is able to phosphorylate a number of different substrate proteins. One of these is phosphorylase kinase (PhK) which, being activated by the phosphorylation, is able to promote a further phosphorylation, that of the enzyme glycogen phosphorylase (GP). By this sequence of events, glucagon and adrenaline are able to stimulate the rate of glucose phosphate production. The cascade containing four enzyme-catalysed steps, permits a substantial amplification of the signal, so that a very small number of hormone molecules can cause a large mobilization of sugar. The molar ratio of three of the enzymes - protein kinase, phosphorylase kinase and phosphorylase, in muscle, being about 1 : 20 : 20 respectively, is in accord with the amplification concept (1).

The kinetic mechanism of phosphorylase from rabbit muscle (30, 31) and that from some other species (32, 33) has been shown to be rapid equilibrium random BiBi:

\[
\begin{align*}
E & \xrightleftharpoons{K_1} EG \\
E & \xrightleftharpoons{K_2} EP \\
E & \xrightleftharpoons{K_3} EGP \\
E & \xrightleftharpoons{K_4} EGP' \\
E & \xrightleftharpoons{K_5} EG'P' \\
E & \xrightleftharpoons{K_6} EG' \\
E & \xrightleftharpoons{K_7} EGP \\
E & \xrightleftharpoons{K_8} EG \\
\end{align*}
\]

where E is the enzyme; P - orthophosphate, P' - glucose-1-P, G and G' - glycogen with n and n-1 glucose residues. \(K_1, K_2\) etc. are the equilibrium constants and \(k_1\) and
$k_2$ are the rate constants for the forward and backward reactions respectively.

The kinetic equation for this mechanism is

\[
\frac{E_0}{v} = \frac{1}{k_1} + \frac{K_4}{k_1(G)} + \frac{K_3 (1 + K_1/K_7)}{k_1(P)} + \frac{K_1}{k_1(G)(P)}
\]

where $E_0$ = total enzyme concentration and $v$ = initial velocity

The above kinetic mechanism has been confirmed by isotope exchange studies (34). The mechanism is unaltered during allosteric transitions, when sigmoidal substrate saturation curves are obtained. The catalytic function of phosphorylase and many of its responses to its regulators are cooperative (7, 18, 35-38).

The model predicts that homotropic and heterotropic cooperativities are interlinked functions. With phosphorylase $b$ separation of these functions has been demonstrated. Several modifications to the original model have been suggested by various authors. Rubin and Changeux (39) have developed the concept of nonexclusive binding as a possibility. Some workers have tried to incorporate additional conformational states other than R and T but complementing them (40-42). Bresler and Firsof (43) preferred additional assumptions taking into account the possibility of non-exclusive binding of ligands. Wang and Tu (42) have noted that their results fit in more satisfactorily with the model of Koshland et al. (44). Mention may be made of the work of Will et al. (45) with hog muscle phosphorylase $b$ for which they developed another equation to explain their results. Soman and Philip have explained their results with a model of 'right and wrong' binding of ligands on the enzyme (46).
Detailed structural studies have been carried out for the enzyme from rabbit muscle. No sulphide bridges have been found in phosphorylase. The subunits of the rabbit muscle enzyme are similar but not identical (47). Electron microscopic measurement has shown that rabbit muscle phosphorylase $b$ consists of 2 ellipsoidal units bound together with measurements of 110 : 65 : 55 Å$^0$ (48). X-ray crystallographic analysis showed the dimensions as 115 : 75 : 60 Å$^0$ (49, 50). The symmetrical association of the dimers gives the tetramer a square shape (51). Tubular shaped crystals have been obtained for phosphorylase $a$ in the presence of protamine (52).

The complete amino acid sequence of the 841 amino acids of the rabbit muscle phosphorylase has been reported by Titani et al. (53). The composition derived from the sequence is as follows: Leu 79, Glu 64, Arg 63, Ala 63, Val 62, Asp 51, Ileu 49, Lys 48, Asn 45, Phe 38, Tyr 36, Thr 35, Gln 31, Ser 29, His 22, Met 21, Try 12 and CySH 9. The data shows that at neutral pH, the positively and negatively charged amino acids are well balanced. However, such a neutralization is not equally distributed. For eg. the N-terminal end has been shown to be composed of essentially basic amino acids (50, 54).

Comparison of the 15-19 amino acids of the N-terminal sequences of $E. coli$ and potato phosphorylases with those from five vertebrate sources and yeast (55) showed that (i) the first amino acid is a hydroxy amino acid (threonine in potato phosphorylase and serine in all other cases), (ii) the non-regulated plant phosphorylases have a free $\alpha$-amino group in contrast to all other phosphorylases studied so far which have a blocked $\alpha$-amino group. The $E. coli$ phosphorylase showed identity with animal phosphorylases only in position 1, 3 and 16. Nakano et al. (56, 57) have shown that the sequence of the potato and rabbit enzyme are very similar except for the remarkable dissimilarity seen at the N-terminal residues. These
phosphorylases are similar in some of the structural and kinetic properties but their control mechanisms are different. The sequence near the PLP site in rabbit phosphorylase has been shown to be homologous to that from yeast (58), potato (57, 59) and *E. coli* (55, 60). The peptides containing SH groups obtained from the rabbit and potato phosphorylases also have highly homologous series (56). From these studies Nakano *et al.* (1980) pointed out that phosphorylase existed originally as a large catalytically active molecule and by gradual mutation a regulatory mechanism was formed within the molecule during the course of evolution.

Phosphorylase has a very complex structure. Each of its two subunits consists of two domains made up of a core of pleated-β-sheets flanked by α-helices. The N-terminal domain includes the subunit boundary, the serine phosphate, the activating AMP and inhibiting glucose-6-phosphate (G6P) binding site, the glycogen storage site and a small part of the catalytic site. The C-terminal domain complements the catalytic site and also contains the neighbouring site where the inhibitory nucleosides and purines bind. The catalytic site lies at the head of a 12-15 Å long tunnel.

In the dimer the two subunits are joined end-to-end at a contact that is tenuous for so large a protein, making up no more than 7% of its surface area in phosphorylase *b* and 10% in *a*. As in haemoglobin, the two fold symmetry axis passes through a waterfilled channel, but in phosphorylase this channel is flanked by two large grottoes, capable of holding about 150 water molecules. Unlike, haemoglobin, the channel contains no binding site for effectors. A view of the dimer perpendicular to the symmetry axis shows that its two sides are very differently constructed. One side is convex with a radius of curvature matching that of the glycogen particle (175 Å). It contains the entrance to the catalytic tunnel and the glycogen storage site, identified by its binding of maltoheptose, a 7-residue oligomer
of α-D-glucose. The side that faces away from the glycogen particle contains the regulatory, phosphorylation sites and the overlapping AMP and G6P binding sites. Most of the binding sites for substrates and effectors are widely separated. A distance of 30 Å separates the catalytic site from the one that binds maltoheptose (the glycogen storage site), which suggests that the enzyme chews away at a piece of polysaccharide chain that is far removed from the piece that attaches the glycogen particle to the enzyme. The closest distance, 15 Å, links the serine phosphate to the AMP binding site, both are over 30 Å from the nearest catalytic site, and the two catalytic sites are over 60 Å from each other. Yet binding of ligands to any of these sites can be shown to affect all the others.

As a first step, X-ray analysis has revealed the changes that phosphorylation of serine 14 induces at the subunit boundary in going from the weekly activated β to the inhibited α structure. The most important of these changes counts in the burial and ordering of the amino-terminal 16 residues and the exposure and disorder of the carboxy-terminal 5 residues in phosphorylase α, and the reversal of these features in phosphorylase β as shown in Fig. 1-3. These movements are accompanied by changes in hydrogen bonding. In β Asp 838 is tied down by a salt bridge to His 36 of the opposite subunit. On transition to α that salt bridge is broken and the histidine rotates about the α-β bond to form a hydrogen bond with the carbonyl oxygen of phosphoserine 14 of the same subunit. The phosphate also forms salt bridge with two arginines, one from the same and the other from the opposite subunit. In the absence of neutralising serine phosphate, the arginines contribute to a cluster of positive charges that expel the positively charged N-terminal peptide from its binding site that spans to two protein subunits. Thus the dominant interactions responsible for the allosteric transition are electrostatic. Other changes in salt bridges and non-polar contacts between subunits follow in train, and those are transmitted to the
Phosphorylase a showing the N-terminal helices, marked N, with the serine phosphates 14, marked by arrows, coordinated to two arginines, one from each subunit and AMP bound firmly between helix α8 and the cap. The C-terminal peptide, marked C, is disordered.

Same view in diagramatic form
Phosphorylase b. The N-terminal residues are disordered and the C-terminal ones are ordered, with Asp 838 hydrogen bonded to His 36 of the opposite subunit. AMP is bound more loosely.

Diagramatic view, with G6P bound at the effector site. The plus sign stands for Lys 9, Arg 10 and Lys 11.
Fig. 1-3 E

Enlarged views of subunit contacts in phosphorylase b (left) and a (right), showing details of some of these interactions.
AMP and G6P binding sites that lie at the subunit boundary only 15 Å from phosphoserine 14. AMP is wedged between an α-helix from one subunit and a non-helical loop from the opposite subunit, referred to as the cap; that loop is separated from phosphoserine 14 by a short helix. Comparison of the two structures shows how dephosphorylation of serine 14 and transition to phosphorylase b weakens both electrostatic and Van der Waals interactions with activator AMP, thus increasing its dissociation constant from the enzyme 100-fold (61, 62). The same changes strengthen binding of the Inhibitor G6P. All these changes take place in the T-structure.

D.Barford and L.N.Johnson have solved the longsought structure of the active R-form of phosphorylase. It emerged from an X-ray analysis of crystals of phosphorylase b grown from ammonium sulfate solution; the sulfate appears to have worked as an activator in place of phosphate at the active site and at the phosphorylation site at serine 14. In these crystals the enzyme is tetrameric, a form of phosphorylase b also found in solution, and the glycogen storage site is buried in a subunit contact. In vivo attachment of the enzyme to glycogen particles causes it to dissociate into dimers.

The allosteric transition consists of rotations of each of the two monomers by 5° about axes pointing in opposite directions normal to the molecular dyad, as shown in Fig. 1-4. The transition affects the helix α 7. They interdigitate and form a bridge between the catalytic sites of neighbouring subunits; Barford and Johnson call them tower helices, because each helix protrudes from its own subunits and penetrates deeply into the neighbouring one (Fig.1-5). The angle between the two helices changes from +20° in the T structure to -80° in the R structure. Each catalytic site is flanked by six loops of chain; some from the N-terminal and some from the C-terminal domain. One of these loops links helix α 7 to α 8; it carries aspartate 283,
Possible changes of quaternary structure in a dimer of two identical subunits related by an axis of two fold symmetry, also called a dyad (symbol on top) (point group C2 or 2). In the simplest transition the left subunit turns anticlockwise about an axis normal to the dyad and pointing towards the observer; the right subunit turns anticlockwise about a colinear axis pointing away from the observer. More generally, the subunits can turn about any pair of axes related by the molecular dyad, for example the ones shown as bold arrows. They point into the picture, away from the observer.
Fig. 1-5

Helices linking active and regulator sites of phosphorylase, in the T-structure seen perpendicular to the molecular dyad.
asparagine 284 and phenyl alanine 285 as its tip, which lock access to the catalytic site when the enzyme is inhibited. Helix $\alpha 8$ reaches from the catalytic site to the AMP binding site of the same subunit. At its C-terminus Arg 309 and 310 form hydrogen bonds with the phosphate of AMP. Finally helices $\alpha 2$ link each of the AMP binding sites to the phosphoserine sites on the opposite subunit. Heterotrophic ones may be transmitted to the regulatory sites by the tower helices and by changes at the subunit contacts of the kind shown in Fig. 1-3.

Goldsmith et al. (1989) have solved the structure of a crystal of phosphorylase $a$ soaked in a solution of orthophosphate and maltopentaose. Phosphate ions were bound at the glycogen storage rather than the catalytic site. The binding of these molecules induced marked changes in the enzyme structure. At the active site the phosphate ion unlocked the 'gate', displaced Asp 283 and formed hydrogen bonds with imino groups at the end of the helix containing residues 133-149. The binding of the phosphates and of the oligosaccharide caused the C-terminal catalytic domain to turn by 1° and shift by 0.5 Å away from the N-terminal regulatory domain, the tower helices moved closer together. Their movement was much smaller than, and different from, the one seen by Barford and Johnson in the R-structure of phosphorylase $b$. Goldsmith et al. suggests that their structure may be an intermediate between the inactive T and the fully active R-structure.

X-ray studies showed how the substrate phosphate, the reaction product G1P and the inhibitory cyclic glucose 1,2-biphosphate bind to phosphorylase $b$ and $a$. The most telling clues were obtained from synchrotron radiation studies of crystals of phosphorylase $b$ activated by AMP and soaked in solutions of the sugar heptenitol and inorganic phosphate. The very intense X-ray beam from the synchrotron storage ring allowed the investigators to take 'Snap shots' of the diffraction pattern at successive stages of the reaction, and to analyse the changes that accompa-
Fig. 1-6

Active site showing pyridoxal phosphate (PLP) and heptulose-2-phosphate (H₂P) hydrogen bonded to the enzyme (63)
nied the gradual accumulation of the product heptulose-2-phosphate that remains bound in the active site as shown in Fig.1-6 (63). The conformations of the protein and coenzyme seen in this figure are similar to those found more recently in the R-structure, where the substrate phosphate is replaced by a sulphate ion and the sugar is absent.

Phosphorylases have been found to contain one molecule of pyridoxal-5'-phosphate (PLP) bound to each subunit of the enzyme. Sucrose phosphorylase however does not contain PLP. This prosthetic group has been shown to be covalently linked to Lys 679 and buried inside a hydrophobic region in rabbit muscle phosphorylase (64, 65). The PLP can be resolved by deformation of the enzyme and trapping it with a reagent like L-cysteine (66). The loss of PLP is accompanied by loss of activity and a tendency to dissociate to monomers at room temperature (67). The quaternary structure of the apoenzyme is different from that of the holoenzyme (68). The apophosphorylase can be reconstituted by the addition of PLP and this process has been shown to be highly temperature dependent. Unlike in other PLP containing enzymes like transaminases, NaBH₄ reduction of the coenzyme does not abolish the catalytic activity of phosphorylase (69). This finding initiated a search of the functional group in PLP that may be participating in catalysis. For this, the apophosphorylase was reconstituted with a number of PLP analogues which differ from PLP at any one of the six positions in the aromatic ring. From these studies it was concluded that except for the pyridine nitrogen and phosphate, all other groups are apparently not participating in the catalytic process (18). Out of a number of 5'-phosphate analogues tested only very closely similar compounds like pyridoxal-5'-methylene phosphate (69) restored catalytic activity. This compound has a pK 7.2 compared to 6.2 for PLP and the reconstituted enzyme showed an alkaline shift in the pH optimum. This supports the earlier assumptions that the phosphate has some
role in catalysis. The finding by Graves and his colleagues (70) that phosphite can activate pyridoxal reconstituted enzyme while pyrophosphate was a competitive inhibitor to both phosphite and glucose-1-P, shows the involvement of the phosphate moiety and its participation in catalysis. The effect of pH on enzymic activity (71) and the earlier study on the dependance of pH on the fluorescence quantum yield of PLP-monomethyl ester (72) shows that the phosphate group (with pK 6.2) may participate in general acid base catalysis.

Since the pyridoxal phosphate has an absorbance maximum at 335 nm, the study of the spectral properties under different conditions with simultaneous activity measurements can yield the structure-function relationship of the coenzyme. Even though the PLP site has been located in the sequence and in X-ray picture, the spectral characteristics predict a more complex environment in which the coenzyme is bound. On reduction of the coenzyme with NaBH₄ the 335 nm bond has been found to shift towards 290 nm (74). When the pH is shifted to the acid or alkaline side, the band again reappears. Since the absorption due to a schiff base at 415 nm, of PLP is only very less in phosphorylase and since the NaBH₄ reduction is highly retarded at neutral pH, it was suggested that the natural form of the enzyme is zwitterionic addition product of some nucleophilic group on the protein with the Schiff base (75). These structure can be represented as follows:

![Diagram representing the spectral properties of PLP and its functional groups under different conditions.](image-url)
Shimomura and Fuki (76) have shown that the different spectrum of apo and holo enzyme shows, in addition to the 335 nm band, a band at 251 nm, which they have assigned to the bound PLP. By studying the factors contributing to the absorption of PLP. Veinberg et al. (77) have shown that the N-atom of the aromatic ring does not have to be invoked in explaining the spectral properties.

Spectral studies on Sepia pharaonis phosphorylase in our laboratory show that the hydrophobic character of PLP site is increased in the presence of cysteine and PLP can exist as the fully protonated form (22).

Pyridoxal phosphate has been shown to be present in phosphorylases of distinct species where the control mechanisms are different. The analysis of amino acid sequence of the PLP site in potato, yeast, E. coli and rabbit muscle phosphorylases shows that they are highly homologous (57, 58, 59). The activity of the potato phosphorylase has also been shown to be highly dependent on PLP (78). As Nakano et al. (59) have pointed out, the very strong conservation of the coenzyme binding site over approximately 1.5 million years is a good support for a catalytic role of PLP. Conservation of protein structure by the expensive way of maintaining a coenzyme, seems to be an impossibility.

In phosphorylase it is not the pyridoxal moiety but the phosphate moiety of pyridoxal-phosphate that activates the substrate (16). It assumes the monoanionic form in the inactive T-state and the dianionic form in the active R-state (79). The binding site for the substrate inorganic phosphate lies next to the pyridoxal phosphate, and both are hydrogen bonded to basic groups of the protein. The binding of heptulose-7-phosphate causes arginine 569 to move from a buried position to
another that is close to the coenzyme and product phosphates, displacing aspartate 283, the 'lock', in the process, thus substituting a buried negative, repulsive charge by a positive one that attracts the phosphates. The movements of Lysine 574 and arginine 569 are also seen in the R-structure and may stabilize the extra ionization of the pyridoxal phosphate that is essential for catalysis. Glutamate 672 has been shown to be essential as a proton acceptor.

Chemical and structural data suggest that catalysis in vivo may involve the steps shown below:

First a proton may be transferred from the coenzyme phosphate to the substrate phosphate. The latter may then act as a general acid, protonating the α-(1-4) glycosidic bond that links the terminal glucose to the glycogen chain. Cleavage of that bond would lead to the formation of an oxocarbonium ion on the free sugar which would be stabilized by the newly formed orthophosphate dianion. Finally, the orthophosphate may transfer its proton to the coenzyme phosphate and simultaneously mount a nucleophilic attach on the oxocarbonium ion, forming glucose-1-phosphate. In this mechanism the coenzyme phosphate plays the dual part of a
Phosphorylase is a more complex allosteric system than either haemoglobin or phosphofructokinase, because both $a$ and $b$ can take up at least two alternative quaternary structures. Sprang and Fletterick suggest that some of the crystal structures analysed by X-rays may be chimeric in the sense that part of each subunit approaches the tertiary R-structure and another part the tertiary T-structure. This may apply to the heptulose-2-phosphate complex of $b$, where the catalytic site has the active and the subunit boundary the inactive form, or to the glucose complex of $a$, where the catalytic site has the inactive structure, while the subunit boundary has a structure close to fully active R.

Phosphorylase $b$ from rabbit muscle was the first enzyme found to have allosteric property. At lower concentration the saturation curve for AMP is sigmoidal. In the presence of metabolites like G6P and ATP sigmoidicity increases. Though the substrate saturation curve for G1P is hyperbolic it is very much sensitive to the presence of the above metabolites. Thus the intracellular concentrations of glucose, glucose-6-P, AMP and ATP influence the catalytic activity of the enzyme.

Phosphorylase $b$ is activated by AMP and inhibited by ATP and glucose-6-phosphate. A number of other nucleotides and sugar derivatives have been found to influence the activity of the enzyme, but since they are not present in tissues they are useful only for structural elucidation and mechanistic studies of the enzyme.

The observations from rabbit phosphorylase $b$ (81) and other sources (20) show sigmoidal activation curve for AMP. Decreasing concentration of AMP increased the $K_m$ for glucose-1-phosphate or orthophosphate. Based on these observations Wyman and Changeux (82) advanced the model for allosteric transitions; the kinetic study with phosphorylase $b$ received special attention mainly to analyse
the properties of this enzyme in the light of the proposed model. However some observations which did not fit in with the prediction of the model of Monod et al. (82) have been mentioned as satisfactorily explained by the model of Koshland, Nemathy and Fitmer (44).

Phosphorylase \(b\) from different sources have been analysed in the light of the model. Here also, general agreement has been recorded. The allosteric constant for rabbit muscle phosphorylase \(b\) was found by Buc and Bu to be 600 (83). For lobster enzyme this constant is 1200 (20). The value of the constant suggests that phosphorylase \(b\) exists mainly in the T state.

Hill's equation, orginally proposed for oxygen binding on haemoglobin can be used for finding the strength of cooperativity of sites (84). The Hill coefficient may be taken as a measure of the strength of cooperativity of sites. Thus in the presence of an inhibitor the \(n\) value will increase and approach the number of binding sites. In phosphorylase \(b\) the number of binding sites for the substrate and for the activator is two.

Extensive work has been done in rabbit muscle phosphorylase \(b\) to evaluate the strength of cooperativity of ligand sites under a variety of conditions. The following observations have been found to satisfactorily fit in with the prediction of the model: the cooperative binding of AMP (81, 85, 86) and the increase in \(n\) value which approaches 2 (81). Under all these conditions the saturation curves for the substrate and AMP are sigmoidal showing clearly that the Michaelis-Menten law is not obeyed. Increasing concentration of AMP or glycogen have been found to decrease the \(K_m\) for glucose-1-phosphate and vice versa, suggesting heterotropic cooperativity of sites (81). So also, the presence of inhibitors decrease the affinity of the enzyme for substrate and activators. According to the model (82), the inhibitors
bind at sites other than the substrate or activator site. Since the reciprocal plots for substrate and activator are non-linear it can not be stated from such plots whether the different ligands bind on the same or different sites.

In addition to the metabolic inhibitors, G6P, ATP and glucose, phosphorylase has been shown to be inhibited by aromatic compounds (87). The inhibition is dependent on the hydrophobicity of the aromatic compounds. The aromatic compounds have been shown to bind on a large hydrophobic region on the enzyme (88). P-nitrophenyl phosphate has been shown to be a competitive inhibitor of AMP for phosphorylase $b$ and is assumed to bind on the same locus where AMP binds (89).

Chemical modification studies of enzyme system reveals the protein functional group. It can also be used as a tool to study allosteric transition. Glycogen phosphorylase has been subjected to chemical modification studies using a number of reagents p-Mercury benzoate, completely dissociated the enzyme into monomers by modifications of the 'SH' groups (90). Using iodoacetamide two out of the 9 SH groups per enzyme monomer were modified without loss of enzyme activity and these groups were shown to be surface exposed (91, 92). Some of the other 'SH' groups reacted very slowly with loss of activity and others were inaccessible to the reagent. Similar results have been obtained when reagents like DTNB (41), NEM (93) and FDNB (94) were employed. Lysl groups have been shown to be essential for the maintenance of enzymic activity and structure. 4-5 amino groups could be modified resulting in totally inactive enzyme (95). Soman and Philip (96) have prepared a desensitised FDNB derivative of phosphorylase in which 1 cysteiny1 and 1 lysl residues were modified. Dinitrophenylation of 1 amino group has also been shown to result in the loss of enzyme activity (97). Glyoxal (98), acetyl imidazole (99), glutaraldehyde and aliphatic aldehyde (100) have also been shown to modify
the enzyme with loss of activity. Modification studies using N-acetyl imidazole has shown that 2 tyrosyl residues are essential for maintaining enzyme activity (101). Nitration also has been shown to modify 2 tyrosyl residues out of 36 per monomer, with loss of activity (102). Modification of phosphorylase b with potassium ferrate resulted in identifying tyrosine 75 as essential for catalysis (103).

Studies on the protection of inactivation by AMP have suggested the nucleotide binding site near this residue. The presence of an essential tyrosine was also shown by reaction of phosphorylase b with potassium-nitrodisulfonate salt (Fremy's radical) (104). Phosphorylase b was rapidly inactivated by 5-diazo 1 H-tetrazole or by 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-P-toluene sulfonate with complete inactivation (105). The loss of activity has been attributed to carboxyl group modification. Histidine residues modified by diethyl pyrocarbonate (106) also resulted in enzyme inactivation. The presence of histidine residues at the substrate binding region is suggested by this study. 2,3-Butane-dione has been employed to study the significance of arginine in catalysis (107). Two types of essential arginine residues, one in the allosteric site and the other in the active site, have been modified.

In fact, phosphorylase a, a naturally occurring desensitized form has entirely different allosteric properties from that of the b form. Homotropic cooperativities of AMP sites are only observed in presence of inhibitors like glucose (108, 109). A glutaraldehyde modified enzyme which is devoid of all homotropic cooperativity but retaining all heterotropic interactions has been prepared (100). Phosphorylase b modified with FDNB in the presence of AMP and orthophosphate has been shown to produce a desensitized enzyme derivative, the analysis of which has shown that lysyl and cysteinyl residues were modified (96). Phosphorylase b, the subunit of which was cross linked by tetroyl bis (glycylazide) has been found to be desensitized
with respect to the AMP binding sites (110) showing that the effect of cross link is to restrict subunit interaction and allosteric transitions of the enzyme.

Phosphorylase \( b \) does not show any activity in the absence of AMP, even at increased concentration of glucose-1-phosphate or orthophosphate. This is one of the major differences from many other allosteric enzymes. If the substrate and activator can bind to the R state, at higher concentration of substrate the enzyme should show considerable activity in the absence of the activator. Yet the presence of glucose-1-phosphate in moderate concentrations has been shown to protect glucose-1-P sites against chemical modification, suggesting that it does bind on its site (95).

The Hill coefficient for AMP sites increases from about 1.4 in the absence of any inhibitor to 1.6 in the presence of ATP (81, 111). But at still higher concentrations of ATP there was no further increase in the \( n \) value (81, 111). According to the model the limit is \( n = 2 \).

Studies on muscle tissues of all vertebrate terrestrial and aquatic animals revealed a similar pattern of control (20, 20, 112-122). The major control device in all these cases is through the interconversion between active and inactive forms of the phosphorylase via phosphorylation or dephosphorylation. Two or more forms of phosphorylase have been reported in blood platelets, rat chloroma, swine kidney (123-125) in the mollusc, crab (21), \textit{Pectan maximus} (126), Brewers yeast, banana leaves, spinach leaves and pea leaves (127, 128, 129) and in a number of other plant tissues (130). Structural and functional difference from the well established rabbit muscle phosphorylase was observed in many of these species. A high concentration of salt is required for activity of liver phosphorylase (131, 132). The human leukocyte \( b \) form is 25\% active in the absence of AMP (133). An additional form of enzyme (phosphorylase c) has been reported in \textit{Pectan maximus} (126). A purified
dimer form of the phosphorylase was not activated by AMP in *Dictyostelium discoideum* (134). A phosphorylase with high specific activity has been isolated from *Neurospora crassa* which can not be considered as a or b form (135). Monomer of phosphorylase b is present in *Mytilus edulis*, which is activated by AMP (23). Though both phosphorylase kinase and phosphatase are present in sepia mantle (22), they do not have any significant role in the control of phosphorylase activity.

The effect of ligands is more significant in phosphorylase b than phosphorylase a (18). Phosphorylase from all terrestrial and aquatic vertebrates are inhibited by G6P, ATP and glucose and activated by AMP (88, 136, 137). Inhibition observed in the presence of these ligands can be considered as allosteric or competitive (partially or completely competitive). Positive or negative cooperativity is also observed in the activation process. The above inhibitors show allosteric inhibition in phosphorylase b from rabbit, rat, man and other vertebrate terrestrial animals (18). No such definite pattern of allosteric inhibition exists for the phosphorylase from marine vertebrates and invertebrates (22, 88, 136, 137). Moreover these ligands are competitive for the enzyme from marine vertebrates and invertebrates. This is very evident in the AMP kinetics in the presence of inhibitors. AMP exhibits positive cooperativity in rabbit phosphorylase (18). Phosphorylase a from the mantle tissue of sepia (22, 137) shows negative cooperativity. Control mechanism of phosphorylase in sepia mantle is totally different (20, 137). *Loligo vulgaris* was selected for a comparative study of glycogen phosphorylases. This invertebrate is an important cephalopod in nerve physiological research.
Loligo vulgaris
Systematic position of this species is as follows (138).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Category</th>
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</thead>
<tbody>
<tr>
<td>Superphylum</td>
<td>Invertebrata</td>
</tr>
<tr>
<td>Phylum</td>
<td>Mollusca</td>
</tr>
<tr>
<td>Class</td>
<td>Cephalopoda</td>
</tr>
<tr>
<td>Subclass</td>
<td>Coleoidea</td>
</tr>
<tr>
<td>Order</td>
<td>Decapoda</td>
</tr>
<tr>
<td>Suborder</td>
<td>Tenthoidea</td>
</tr>
<tr>
<td>Family</td>
<td>Loliginidae</td>
</tr>
<tr>
<td>Type</td>
<td>Loligo</td>
</tr>
</tbody>
</table>

The genus Loligo has a world wide distribution in the warmer seas. The *Loligo vulgaris* otherwise called squids, are the most active of the cephalopods occurring in coastal water, in deeper water and in the abysses. The squid has a tapering body, hence the nick name 'sea arrow'.

The mantle forms the thick, muscular and protective envelope, enclosing the visceral hump and the mantle cavity. The conical projections of the mantle, one on each side of the animal forms the fins. Ventrally, the free mantle edge forms a loose collar around the neck region, thus leaving a circular opening, through which water enters the mantle. A conical muscular tube projecting beyond the collar, beneath the head, is the funnel, through which the water of mantle cavity is expelled out. The mantle and funnel form the chief locomotory organs. The customary mode of the locomotion is slow swimming by the undulating movements of the fins, during which the arms are closely extended in front to serve for steering. But, when the animal is excited, the mantle collar closes tightly around the neck and the water is forcibly ejected through the siphon, so that the animal is propelled in the opposite direction like a rocket by the principle of jet propulsion. The rocket-like tapering body enables it to dart through water with lightning like speed.
The squids are invertebrates and low in evolutionary status from vertebrates.

Through evolution, the vertebrates have acquired the mechanisms of regulation of glycogen degradation within the phosphorylase by gradual mutation, as evidenced by the amino acid sequence analysis in a number of distinct animal species and plants (55-57). It appears that the higher demand on glycogen for energy in loligo mantle is met by maintaining a high concentration of AMP rather than evolving a structurally more active form of phosphorylase.

The above facts reveal that the nature of phosphorylase is linked with the energy requirement and/or evolutionary status of the animals. A systematic comparison of properties of phosphorylases from marine sources is presented in this thesis.