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The Role of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase Catalysis†

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Pyridoxal 5′-Phosphate in Phosphorylase

Glycogen phosphorylase is found in every species from unicellular organisms and bacteria to the complex tissues of higher plants and mammals where it plays a key role in carbohydrate metabolism. The best studied example of the highly regulated mammalian glycogen phosphorylases is the rabbit muscle phosphorylase, which was isolated and characterized by G. T. Cori and C. F. Cori [cf. Brown and Cori (1961)], who also described two forms (Cori & Green, 1943): one of which, phosphorylase *a*, was active without 5′-AMP and the other, phosphorylase *b*, was inactive unless 5′-AMP was added. Shortly after Baranowski and colleagues (Baranowski et al., 1957) in Cori's laboratory discovered pyridoxal 5′-phosphate in rabbit skeletal muscle phosphorylase, Fischer et al. (1958) reported that the bond linking the 4′-aldehyde group of pyridoxal 5′-phosphate to the ϵ-amino group of a lysine of the enzyme could be reduced with sodium borohydride without substantial loss of activity. Since all pyridoxal 5′-phosphate dependent enzymes in amino acid metabolism require a functional aldehyde group of the cofactor for activity and become inactive on reduction, it became clear at once that if pyridoxal 5′-phosphate in phosphorylase should also participate in catalysis, it must do so in a manner different from that operative in all the other known vitamin B_6 dependent enzymes (Snell & DiMari, 1970). This is also borne out by the different disposition and geometry of pyridoxal 5′-phosphate bound to aspartate aminotransferase, the only other pyridoxal 5′-phosphate containing enzyme whose 3-D structure is known (Kirsch et al., 1984).

Ever since pyridoxal 5′-phosphate was shown to be indispensable for activity of rabbit skeletal muscle phosphorylase, the role of this cofactor in phosphorylase has puzzled enzymologists. Shaltiel et al. (1966) found that the cofactor could be removed quantitatively once the protein was deformed with imidazole citrate. Removal of pyridoxal 5′-phosphate was accompanied by complete loss of enzymatic activity, but activity was restored on reconstitution with pyridoxal 5′-phosphate (Hedrick et al., 1966). This made it possible to test more than 30 pyridoxal 5′-phosphate analogues modified in every single position of the substituted pyridine ring for their ability to reactivate apophosphorylase [see Graves and Wang (1972)]. These systematic studies gave the first indication that the 5′-phosphate group of the cofactor was the most likely candidate for participation in catalysis, since only pyridoxal 5′-phosphate analogues that contribute a reversibly protonatable dianion, like $-OPO_3^{2-}$ or $-CH_2PO_3^{2-}$, in the 5′-position were able to reconstitute an active enzyme. This generalization was supported by NMR spectroscopy (Feldmann & Hull, 1977), which revealed that the 5′-phosphate of pyridoxal 5′-phosphate in the nonactivated form of phosphorylase *b* is a monoprotonated phosphate species (form I), whereas in phosphorylase activated allosterically (or by phosphorylation) the phosphorus NMR signal could be assigned to the dianionic form (form III) [see also Hörl et al. (1979)]. This significant finding was corroborated by phosphorus NMR studies with potato (Klein & Helmreich, 1979) and *Escherichia coli* maltodextrin phosphorylase (Palm et al., 1979) in both of which the activity is neither allosterically nor covalently controlled. Hence, as was to be expected, the 5′-phosphate group of the cofactor was in the dianionic form (form III) in these phosphorylases (see Figure 1). More recently it was shown that replacement of Glu 672[1] by Asp or Gln by site-directed mutagenesis blocks the transition between protonation states I and III and results in a 1000-fold decrease of k_{cat} (Schinzel and Palm, unpublished experiments). On the basis of these and additional NMR data in the presence of maltoheptaose or glucose (see Figure 1; Klein & Helmreich, 1979; Helmreich & Klein, 1980), it was suggested that the 5′-

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[1] Sequence numbers for rabbit muscle phosphorylase are used (see Table III).

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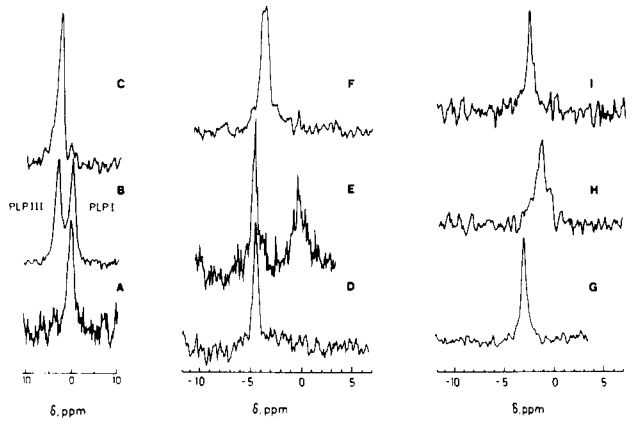


FIGURE 1: ^{31}P NMR spectra of pyridoxal 5′-phosphate in phosphorylases. The dianionic state ($PLOPO_3^{2-}$) and monoprotonated state ($PLOPO_3H^-$) are assigned to the low- and high-field forms, respectively. (Left panel) Rabbit muscle phosphorylase *b*: (A) no ligands, pH 7.8; (B) 0.4 mM AMP-S (adenosine 5′-thiophosphate) and 50 mM arsenate, pH 8.0; (C) 1.1 mM AMP-S, pH 8.1. (Middle panel) Potato phosphorylase: (D) no ligands, pH 6.5; (E) 1 mM maltoheptaose, pH 6.5; (F) 15 mM maltoheptaose and 100 mM arsenate, pH 6.5. (Right panel) *E. coli* maltodextrin phosphorylase: (G) no ligands, pH 6.9; (H) 100 mM maltoheptaose, pH 6.4; (I) 50 mM maltoheptaose and 100 mM arsenate, pH 7.0. The spectra are reproduced from Feldmann and Hull (1977), Hörl et al. (1979), Klein and Helmreich (1979), Palm et al. (1979), Helmreich and Klein (1980), and Feldmann et al. (1978).

phosphate group of pyridoxal 5′-phosphate functions in phosphorylase as a proton donor–acceptor shuttle in a general-acid–base catalysis (Feldmann et al., 1978; Helmreich & Klein, 1980; Klein et al., 1981). However, this proposal was not accepted by Graves (Parrish et al., 1977; Chang et al., 1983) and by Madsen and their colleagues (Withers et al., 1981a, 1982). Parrish et al. (1977) had shown that phosphorylase reconstituted separately with pyridoxal and fluorophosphate shows significant activity, e.g., about one-fifth of that of the native enzyme. On the basis of these experiments, they argued that this would be incompatible with a proton donor–acceptor function for the 5′-phosphate of the cofactor, since fluorophosphate has a $pK \approx 4.8$ and cannot become protonated at pH 6.8 where phosphorylase is active. However, in phosphorylase catalysis fluorophosphate cannot replace a protonatable "substrate phosphate anion" (see later). Moreover, phosphate or fluorophosphate does not bind in the pyridoxal-reconstituted phosphorylase to the same position to which these anions bind when linked covalently to the 5′-OH of pyridoxal (Oikonomakos et al., 1987). It was therefore not surprising that phosphorylase reconstituted with pyridoxal 5′-fluorophosphate was inactive (Klein et al., 1982), since the latter, in contrast to pyridoxal 5′-phosphate, has only one ionizable site (Klein et al., 1984a). Graves and colleagues (Chang et al., 1983; Soman et al., 1983) still maintain that the 5′-phosphate of the cofactor is mainly a structural determinant holding other reactive groups at the active site in the right orientation for catalysis. On the other hand, Madsen, Fukui, and colleagues (Withers et al., 1981a, 1982; Takagi et al., 1982) proposed a different role for the dianionic 5′-phosphate of the cofactor in which a "constrained" dianion functions as an electrophilic catalyst. But aside from that crucial difference of opinion, Madsen, Fukui, and their colleagues (Withers et al., 1981a,b, 1982; Takagi et al., 1982; Madsen, 1986) did clearly recognize the important role of noncovalent interactions between the 5′-phosphate group of the cofactor and the substrate phosphates.

The concept, which we shall now describe in detail, was profoundly influenced by the direct experimental proof of protonation of glycosylic substrates in the presence of phosphate by phosphorylase (Klein et al., 1982). This led us to postulate that the 5′-phosphate of pyridoxal 5′-phosphate transfers its proton to another "substrate" phosphate which in turn protonates the glycosidic bond. But a precise understanding of the role of pyridoxal 5′-phosphate in phosphorylase catalysis did actually emerge only recently from studies employing a variety of approaches reaching from classical enzymology to NMR spectroscopy and X-ray crystallography. Thus, it was the use of glycosylic substrate analogues that not only made possible the conception of a catalytic mechanism but also allowed for the visualization of enzyme–product complexes in the crystal by high-resolution X-ray diffraction (McLaughlin et al., 1984; Hajdu et al., 1987).

Reaction of Glycogen Phosphorylase

α-Glucan phosphorylases catalyze the sequential phosphorolytic degradation of oligo- or polysaccharides and the formation of α-1,4-glycosidic bonds in poly- or oligosaccharides from glucose 1-phosphate:

$$(\text{glucose})_n + P_i \rightleftharpoons (\text{glucose})_{n-1} + \alpha\text{-D-glucose 1-phosphate}$$

The reaction is readily reversible in vitro (K_{eq} = P_i/glucose 1-phosphate = 3.6, pH 6.8).

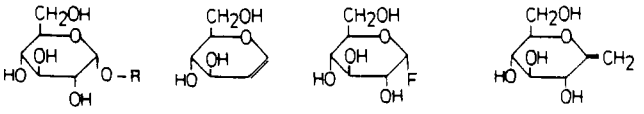
Early proposals of a catalytic mechanism centered around the idea that the reaction proceeds with retention of configuration of the α-glycosidic bond involving sequential double inversion, whereby the glucose residue passes through a hypothetical intermediate (Brown & Cori, 1961). Such an intermediate was shown to be formed in sucrose phosphorylase, which does not contain pyridoxal 5′-phosphate but likewise catalyzes reversible transfer of glucosyl units with retention of configuration between glucose 1-phosphate and sucrose, but not oligo- or polysaccharides (Doudoroff, 1961; Voet & Abeles, 1970). However, in glycogen phosphorylase, lack of phosphate exchange between glucose 1-phosphate and $[^{32}P]P_i$ (Cohn & Cori, 1948) and random bi–bi kinetics (Chao et al., 1969; Engers et al., 1969) were not in favor of a covalent glucosyl intermediate. The finding by Kokesh and Kakuda (1977) of an exchange of the sugar ester oxygens and the phosphoryl oxygens of α-D-glucose 1-phosphate catalyzed by potato phosphorylase in the presence of cyclodextrin could be interpreted in favor of a covalent glucosyl intermediate, but does not exclude a mechanism where a stabilized glucosyl cation is formed.

D-Glucal, which yields a 2-deoxyglucosyl moiety on protonation, would seem to be especially suited to form covalent bonds with the enzyme in the absence of an acceptor (Legler et al., 1979), but D-glucal did not give rise to a stable derivative of glycogen phosphorylase (Helmreich & Klein, 1980; Klein et al., 1981, 1984a). Although no evidence for an enzyme-bound intermediate was obtained, the study of the D-glucal reaction revealed an obligatory requirement for phosphate ions for substrate activation, and this was the starting point of an extended study with glycosylic substrates.

Studies with Glycosylic Substrates

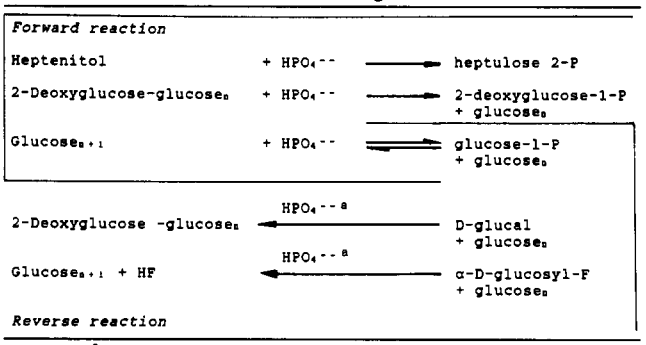
α-Glucan phosphorylases have in common with carbohydrases the fact that they are glycosyl transferases. This could explain why phosphorylases accept glycals as substrates and catalyze the reaction with them at significant rates (Klein et al., 1982, 1984a,b). Glycosylic substrates are compounds of nonglycosidic structure with the potential anomeric carbon linked via an electron-rich bond (Table I). They were introduced by Hehre et al. (1973) to the study of glycosylases.

Table I: Glycosylic Substrate Analogues Reactive with Phosphorylase[a]



[a] Cf. Klein et al. (1982, 1986) and Palm et al. (1983).

Table II: Reaction Pathways Catalyzed by Phosphorylase with Natural Substrates and Substrate Analogues



[a] HPO_4^{2-} is required for substrate activation but does not participate as a reactant.

Formation of a transferable glycosyl residue from a glycosylic substrate requires a stereoselective protonation which, in the case of endocyclic or exocyclic enitols such as D-glucal or heptenitol, leads to products retaining the activating proton. Glycosylic substrates are therefore ideally suited to the study of a protonation-type reaction mechanism with participation of the 5′-phosphate of the cofactor.

Reaction with D-Glucal and α-D-Glucosyl Fluoride

A major conclusion drawn by Lehmann and Schröter (1973), Hehre et al. (1977), and Chiba et al. (1988) from their studies with carbohydrases and glycals such as D-galactal and D-glucal was that one carboxyl group in the enzyme is acting as a general acid which protonates the glycosylic (or the glycosidic) bond while another carboxyl group acts as a nucleophile or a base stabilizing the charge and determining the stereospecific course of the reaction. Our results with phosphorylases and glycosylic substrates are in agreement with these conclusions, as far as a requirement for a general acid and a nucleophile is concerned. However, an important difference between phosphorylases and carbohydrases is that the former require, in addition to the first substrate (which can be the same as that for carbohydrases), a primer and phosphate anions, whereby arsenate is the only anion that can replace phosphate (see Table II). In order to distinguish these anions from the 5′-phosphate dianion of pyridoxal 5′-phosphate, we have named the former the "substrate" or "mobile" phosphate anion (Klein et al., 1986). Besides D-glucal, α-D-glucosyl fluoride was also accepted as a phosphorylase substrate in the presence of phosphate, but it reacted at a slow rate (Palm et al., 1983). The dependence of the phosphorylase reaction with glycosylic substrate analogues on both pyridoxal 5′-phosphate and mobile phosphate anions raised two questions: first, whether the mobile phosphate is directly involved in the protonation of glycosylic and glycosidic substrates, and second, how pyridoxal 5′-phosphate might assist the mobile phosphate in its action.

To answer these questions, the steric and functional consequences of a substitution of glucose 1-phosphate by D-glucal, heptenitol, or α-D-glucosyl fluoride and phosphate were traced by "model building" (not shown) of substrate complexes with phosphorylase *b*. In accordance with the structural information of McLaughlin et al. (1984) and Barford et al. (1988), analysis of the catalytic site showed that there is scope for the substrate phosphate in closely similar positions to act as a proton donor either to heptenitol or to glucal as proposed in Figure 2. The equally variable orientation of the phosphate supports its role as a general acid in protonating the electron-rich glycosylic bond of the substrate, whereby pyridoxal 5′-phosphate assists the mobile phosphate because the latter can form a proton shuttle with the cofactor phosphate.

Reaction with D-Glucoheptenitol

The reaction of phosphorylase with an exocyclic glycal, D-glucoheptenitol (heptenitol), which Hehre et al. (1980) and Schlesselmann et al. (1982) had introduced as a prochiral enzymic substrate and as a glycosyl donor for α-glucosidase, seemed at first sight to proceed like the reaction with the endocyclic D-glucal. However, a closer look at the product revealed that the primer was not elongated in the course of the reaction (Klein et al., 1986). Thus, heptenitol is a substrate for only one direction, e.g., glucosyl transfer to phosphate yielding heptulose 2-phosphate (see Table II). It now becomes clear, whatever the direction may be, that in each case the presence of phosphate is obligatory. The fact that heptulose 2-phosphate formed from heptenitol is a "suicide inhibitor" that binds with high affinity (K_i = 2–14 μM) and the absence of a requirement for primer made heptenitol a welcome tool for the X-ray crystallographers. It enabled them to determine for the first time the structure of the catalytic site in an active (R-state) conformation (McLaughlin et al., 1984). Formation of heptulose 2-phosphate in the crystal demonstrated unequivocally direct interactions of the phosphate of the cofactor with the substrate phosphate as required by the "general-acid" mechanism (Hajdu et al., 1987). Such a direct interaction was also shown to occur by ^{31}P NMR (Klein et al., 1984a) while the proximity of the phosphates was apparent from work of Madsen, Fukui, and colleagues (Withers et al., 1981a; Takagi et al., 1982). X-ray crystallographic structure determination of the phosphorylase–heptulose 2-phosphate complex (McLaughlin et al., 1984; Hajdu et al., 1987) also clarified two other important points: first, it showed that there is no nucleophile or carboxylate near the 1β-position of the glycosylic substrate that could stabilize a transient oxocarbonium ion (or a covalent intermediate) in the case of a double inversion; second, there are no positive charges positioned in such a manner that they could constrain the 5′-phosphate dianion of pyridoxal 5′-phosphate as one might have expected on the basis of the electrophilic mechanism postulated by Madsen and his colleagues [see Withers et al. (1982)].

Figure 2 summarizes the reactions for glycosylic substrates and phosphorylase. Figure 2, mechanism I, shows the D-glucal reaction: It is proposed that orthophosphate (or arsenate) protonates the endocyclic double bond from below C-2 of the pyranose ring and, subsequently, participates in charge stabilization of the incipient 2-deoxyglucosyl carbonium ion. Nucleophilic attack by an incoming primer glucosyl on the carbonium ion terminates the reaction. Before the next turnover, the mobile phosphate must be recharged (reprotonated) by the protonated phosphate anion of pyridoxal 5′-phosphate. The reaction with heptenitol in the direction of phosphorolysis is shown in Figure 2, mechanism II. In this sequence of reactions the mobile phosphate anion stays in place and functions subsequently as a nucleophile. Accordingly, the reaction heptenitol + P_i → heptulose 2-phosphate is analogous

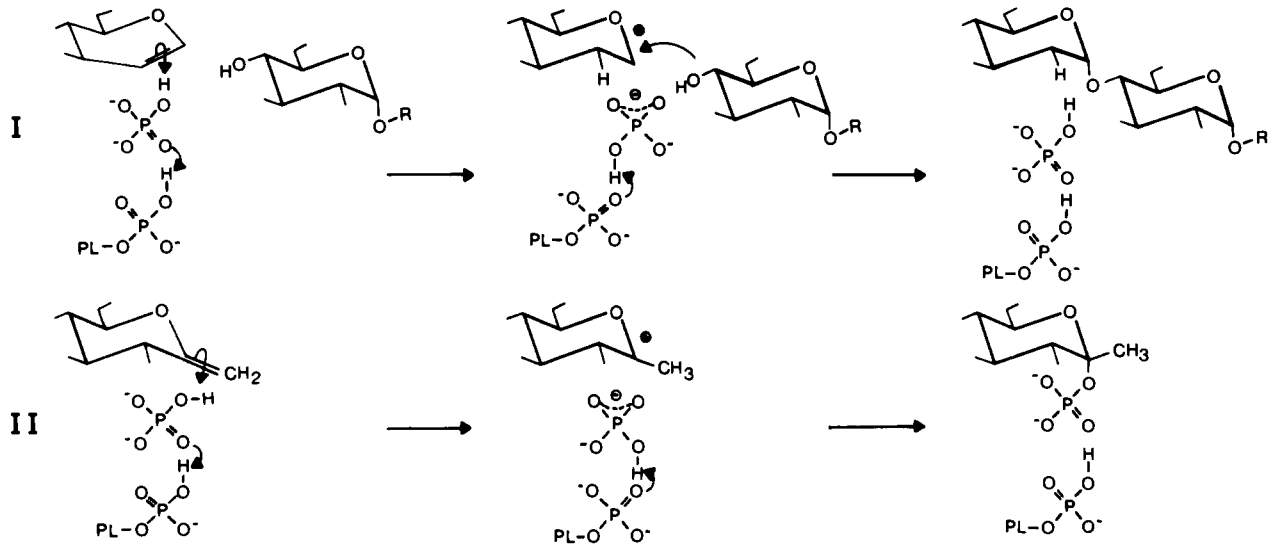


FIGURE 2: Activation of phosphorylase substrates by protonation: (I) D-glucal; (II) heptenitol. PL = pyridoxal; R = α-1,4-oligosaccharides.

Table III: Evolutionary Conserved Sites in Phosphorylases[a]

*Rab*P	131-LGNGGLGRLA-	283-DN-
*Yea*P	-LGNGGLGRLA-	-DN-
*Pot*P	-LGNGGLGRLA-	-DE-
*Eco*M	-LGNGGLGRLA-	-DN-

*Rab*P	309-RR-	338-NDTHP-	373-AYTNHTVLPEALE-
*Yea*P	-RR-	-NDTHP-	-AYTNHTVLPEALE-
*Pot*P	-SR-	-NDTHP-	-AYTNHTVLPEALE-
*Eco*M	-RR-	-NDTHP-	-AYTNHTVLPEALE-

*Rab*P	452-VNGV-	483-TNGITPRRW-	566-QVKRIHEYKRQ-
*Yea*P	-VNGV-	-TNGITPRRW-	-QVKRIHEYKRQ-
*Pot*P	-VNGV-	-TNGVTPRRW-	-QVKRIHEYKRQ-
*Eco*M	-VNGV-	-TNGITPRRW-	-QIKRLHEYKRQ-

*Rab*P	648-YRVSLAE-	661-DLSEQISTAGTEASGTGNMKFMLNG-685
*Ssu*P		-MKFMLNG-
*Yea*P	-YNVSKAE-	-DLSEHISTAGTEASGTSNMKFVMNG-
*Pot*P	-YNVSVAE-	-DLSEHISTAGMEASGTSNMKFAMNG-
*Eco*M	-YCVSAAE-	-DISEQISTAGKEASGTGNMKLALNG-

[a] Conserved amino acid residues are related to proposed substrate (■), pyridoxal 5′-phosphate (▼), or effector (○) sites in glycogen phosphorylase *b* (*Rab*P), yeast phosphorylase (*Yea*P), potato tuber phosphorylase (*Pot*P), maltodextrin phosphorylase from *E. coli* (*Eco*M), and dogfish phosphorylase (*Ssu*P). K680 contains the phosphopyridoxyl residue and is marked by an asterisk and bold type. Underlined sequences correspond to fluorescent phosphopyridoxyl peptides isolated from $NaBH_4$-reduced phosphorylases (see text). The one-letter amino acid code is used. Differences from the *Rab*P sequences are italicized.

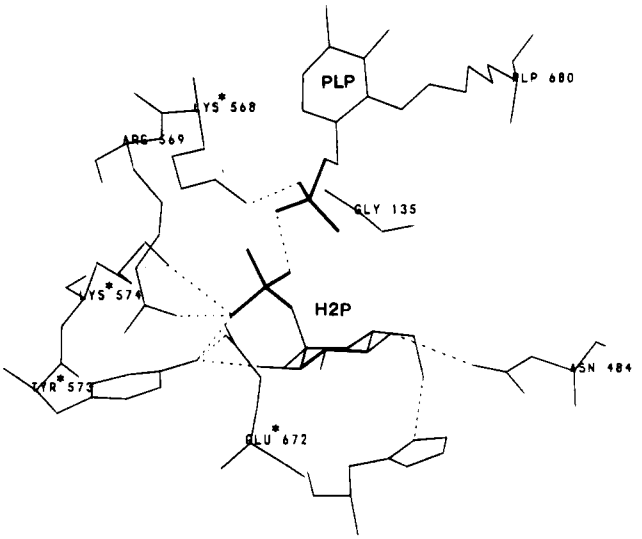


FIGURE 3: The active site in phosphorylase *b* [adapted from Hajdu et al. (1987) for the unrefined heptulose 2-phosphate–phosphorylase *b* complex]. The picture shows polar contacts and hydrogen bonds in the environment of heptulose 2-phosphate (H2P) and pyridoxal 5′-phosphate (PLP) bound to Lys 680. The phosphates are set off by dark bonds. Amino acids marked by asterisks indicate positions that were altered by site-directed mutagenesis as is discussed in the text.

to an addition without discharge of the product, a fact of which Johnson and colleagues took advantage when they studied the heptenitol reaction in the crystal (McLaughlin et al., 1984; Hajdu et al., 1987).

Site-Directed Mutagenesis with *E. coli* Maltodextrin Phosphorylase

Work in Fischer's laboratory revealed an impressive similarity of sequences at the pyridoxal 5′-phosphate site in rabbit muscle (Forrey et al., 1971), dogfish (Cohen et al., 1973), and yeast (Lerch & Fischer, 1975) phosphorylases. Although this argued for a catalytic role of pyridoxal 5′-phosphate, the evidence based on the complete sequences of rabbit muscle glycogen phosphorylase (Titani et al., 1977; Nakano et al., 1986) and *E. coli* maltodextrin phosphorylase (Schächtele et al., 1978; Palm et al., 1985, 1987a) showed that more than 30 "conserved" residues are spread over the entire sequence with the exception of the N-terminal or "regulatory" tail (Table III). But taking the crystallographic evidence on substrate and effector binding sites into consideration, a footprint of conserved amino acid residues, which are functionally relevant, can be traced from bacteria to yeast (Hwang & Fletterick, 1986), higher plants (Nakano & Fukui, 1986; Nakano et al., 1978), and mammals (Table III). On the basis of this information and the structure of the phosphorylase–heptulose 2-phosphate complex shown in Figure 3, the amino acids most likely to be involved in catalysis were selected and replaced by site-directed mutagenesis.

The cloned *mal*P gene from *E. coli* (Raibaud et al., 1983; Bloch & Raibaud, 1986) provided an opportunity to generate site-directed mutants of maltodextrin phosphorylase (Palm et al., 1987a,b; Palm & Schinzel, 1989; Schinzel and Palm, unpublished experiments). Since a 3-D structure of *E. coli* maltodextrin phosphorylase is not available, its structure was inferred from the crystal structure of rabbit muscle phosphorylase, and the sequences were aligned accordingly. All mutated amino acids were near the pyridoxal 5′-phosphate and sugar phosphate binding sites in a region corresponding to the active site in rabbit skeletal muscle phosphorylase *b*.[1]

A prominent arrangement of basic residues, Lys 568, Arg 569, and Lys 574, is located close enough for interactions with

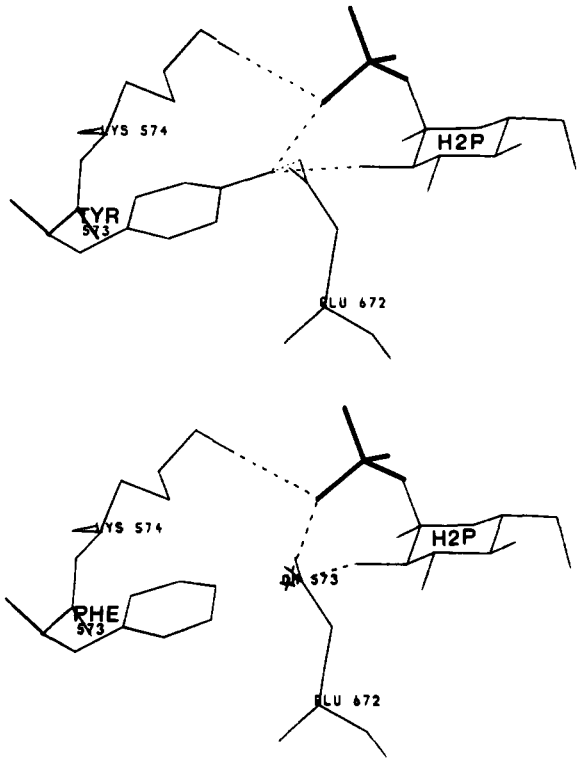


FIGURE 4: View of the substrate binding site of phosphorylase showing the network of polar contacts before and after a substitution by mutagenesis of Tyr 573 to Phe. OH 573 is the position of a water molecule derived from model building (see legend to Figure 3).

the phosphates of pyridoxal 5′-phosphate and the substrate phosphate (Figure 3). Lys 568, which is in hydrogen-bond distance to the cofactor phosphate (Oikonomakos et al., 1987), was substituted for by uncharged amino acids of smaller or comparable size, e.g., Ser or Gln. A 200–1000-fold reduction of k_{cat} in the Lys 568 mutant enzyme may be rationalized by assuming that Lys 568 helps to position the phosphates of pyridoxal 5′-phosphate and the substrate in an orientation to allow for catalysis (see Figure 3). The removal of a neutralizing charge from one of the two phosphate groups could explain the 20–40-fold increase in K_m for P_i in the mutant enzyme. In the case of Arg 569 an interaction with the substrate phosphate is indicated by a reorientation of the side chain of Arg 569, which swings in the phosphorylase *b*–heptulose 2-phosphate complex releasing the whole loop Pro 281 → Gly 288 in order to make contact with the substrate phosphate (Hajdu et al., 1987; Johnson et al., 1989; see also Figure 3). Concerning Lys 574, we have not been able to recover a viable phosphorylase mutant.

We have also considered that the binding interactions between phosphates and positively charged groups could be altered by negatively charged amino acid residues in the vicinity. Therefore, Glu 672, the only acidic amino acid residue within this region, was replaced by the isoionic Asp or the isosteric Gln. The carboxyl group of Glu 672 is close to the 2-OH and 3-OH of the glucopyranose ring of the substrate, and Glu 672 is moreover within H-bonding distance of the OH or Tyr 573 (see Figure 4) and is part of the hydrogen-bond network connecting the substrate phosphates. All mutations of Glu 672 lowered the k_{cat} value by several orders of magnitude without changes of the K_m values for maltoheptaose and P_i but with small but significant changes in the case of glucose 1-phosphate. Hence, interactions with the substrate in the ground state are not changed much when Glu 672 is replaced, whereas the much more pronounced decrease in k_{cat} in the mutant enzyme signals the large effect of the charged carboxyl group of Glu 672 on the binding of the substrates in the transition state and points to a role in the protonation of the 5′-phosphate of pyridoxal 5′-phosphate (see above).

An interaction of Tyr 573 with the glucopyranosyl moiety of various substrates is reflected in the K_m changes resulting from the Tyr 573 → Phe replacement. The more impressive differences in affinity between D-glucal and glucose 1-phosphate both in the wild-type and in the Tyr 573 → Phe mutant enzyme are indicative of the role of additional hydrogen bonds to the 2-OH of the glucose in the case of glucose 1-phosphate (Klein et al., 1984a; Street et al., 1986).

Model building with Phe 573 and water replacing Tyr 573 shows that H_2O can substitute for the Tyr-OH for most of its hydrogen bonds in connection with the substrate (see Figure 4). The H_2O must, however, be properly placed next to the hydrophobic Phe and exactly at the place normally occupied by the OH of tyrosine [cf. Oxender and Fox (1987) and Benkovic et al. (1988)]. The formation of a water site in the mutant enzyme could be responsible for a significant increase in "error" frequency as expressed in terms of the ratio of phosphorolysis/hydrolysis: The ratio of the formation of glucose 1-phosphate versus glucose is 9000:1 for the wild-type maltodextrin phosphorylase but 500:1 for the Tyr 573 → Phe mutant. Thus, in this respect the mutant phosphorylase behaved more like sucrose phosphorylase than glycogen phosphorylase (Doudoroff, 1961; Silverstein et al., 1967; Klein & Helmreich, 1985).

A Mechanism for the Phosphorylase Reaction

Stereospecific protonation of D-glucal provided the first direct proof of a general- (Brønsted-) acid-type activation mechanism for the phosphorylase reaction in direction of synthesis and gave considerable support for our earlier suggestions and proposal (Kastenschmidt et al., 1968; Feldmann et al., 1978; Helmreich & Klein, 1980) of a catalytic role for the phosphate group of pyridoxal 5′-phosphate in a proton shuttle. Subsequently, heptenitol was found to react in a similar fashion, but exclusively in the direction of phosphorolysis (Table II). Hence, it is now established that the reactions in both directions are initiated by activation of the substrate by protonation. This information allowed the presentation of a plausible reaction mechanism (see Figure 5): In the forward direction, e.g., phosphorolysis of α-1,4-glycosidic bonds in oligo- or polysaccharides, the reaction is started by the protonation of the glycosidic oxygen with orthophosphate functioning as the general acid, e.g., $H_2PO_4^-$. Subsequently, the glycosidic bond is cleaved and the incipient oxocarbonium ion is stabilized on the front side by the phosphate dianion. The reaction proceeds by nucleophilic attack of the phosphate on the carbonium ion, which might force the orthophosphate to readjust its position prior to bond formation. The product, glucose 1-phosphate, dissociates and is replaced by a new incoming phosphate. In the reverse direction, α-1,4-glycosidic bond formation and primer elongation, the reaction starts with the protonation of the "acid-labile" phosphate of glucose 1-phosphate, which weakens the glycosidic bond and promotes stabilization of the complex of the carbonium ion and the phosphate anion. A sequential double inversion of configuration is not required, nor is it possible, since the structural prerequisites are missing (see Figure 3). In this case the stabilization by phosphate assures retention of configuration. Once the phosphate is separated, it might also serve as a hydrogen-bond acceptor for the 4-OH of the terminal nonreducing glucose residue of the oligosaccharide primer. This would facilitate a nucleophilic attack of the primer at C_1. The elongated primer must dissociate

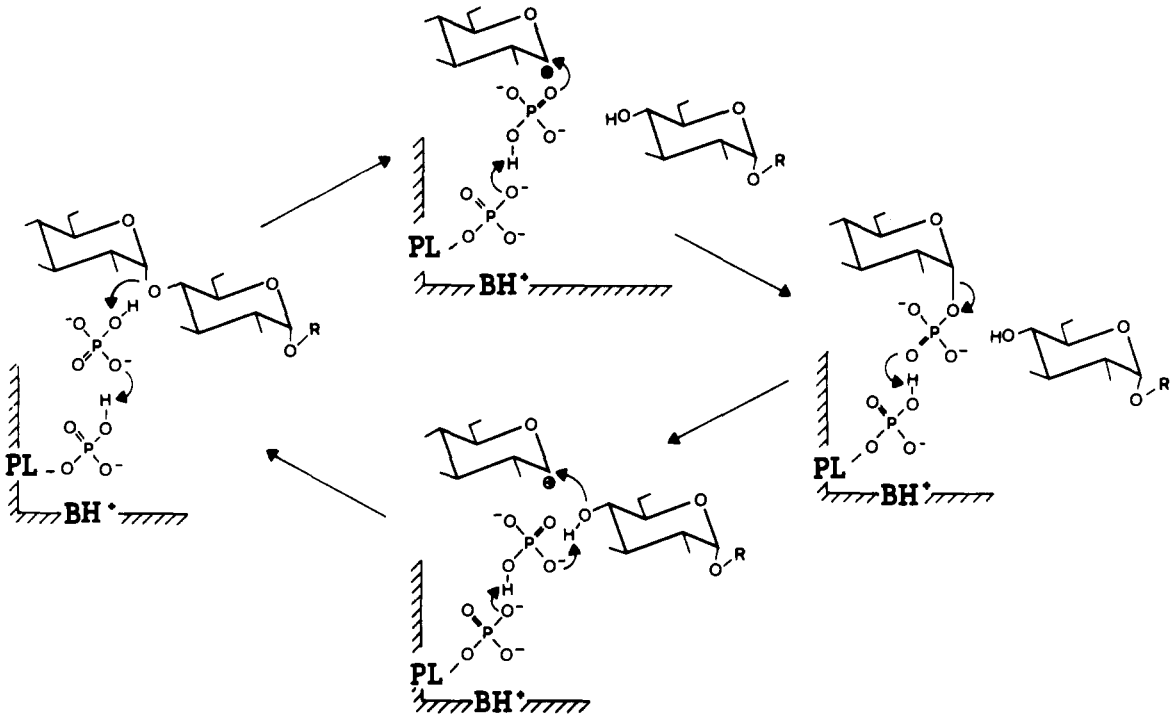


FIGURE 5: Catalytic mechanism of α-glucan phosphorylases. The reaction scheme accounts for the reversibility of phosphorolysis of oligosaccharides (R) in the presence of orthophosphate (upper half) and primer-dependent synthesis in the presence of glucose 1-phosphate (lower half). PL = enzyme-bound pyridoxal; BH^+ = a protonatable general base contributed by the enzyme protein.

before a new round of synthesis can begin. The action pattern of glucose addition to the primer is in agreement with this mechanism (Palm et al., 1973).

The mobile phosphate anion acquires its proton from the phosphate of pyridoxal 5′-phosphate [cf. Klein et al. (1986)]. To accomplish this, the phosphates of the cofactor and the substrate must approach each other within a hydrogen-bond distance (2.8–3.0 Å) corresponding to a distance of the phosphorus atoms of 4.7–5.3 Å. This was actually shown to be the case, since the phosphorus atoms in the phosphorylase–heptulose 2-phosphate complex are only 5.1 Å apart. On the other hand, the mechanism proposed by Madsen and Fukui (Withers et al., 1981a, 1982; Takagi et al., 1982) is based on a pyrophosphate-like alignment of phosphates in analogy with a phosphoryl-transfer reaction as in the case of adenylate kinase [cf. Pai et al. (1977)]. Accordingly, this type of electrophilic mechanism would require an even closer approach of the cofactor phosphate and the substrate phosphate and a much tighter coordination which in turn would make it difficult, if not impossible, to explain the ready reversibility of the phosphorylase reaction.

A mechanism of the kind proposed in Figure 5, although chemically plausible, would not be expected to proceed without assistance from the enzyme protein. This is exemplified by stereospecific protonation giving rise to products, all with glycosidic bonds having α-configurations. Moreover, the role in catalysis of certain amino acid residues of the enzyme, localized by X-ray crystallography at the active site, is impressively demonstrated by site-directed mutagenesis. The basic amino acids Lys 574 and Arg 569 have a decisive role to play in productive binding of the substrate phosphates, indicated by our inability to isolate an active mutant phosphorylase in which Lys 574 was replaced by Ser or Gln and by evidence of others that the interaction between the phosphates and Arg 569 triggers the T → R transition in the course of activation (Dreyfus et al., 1980; Hajdu et al., 1987; Barford et al., 1988). On the other hand, the consequences of substitution of Lys 568 and Glu 672 support the proposed role of the phosphate of the cofactor and the substrate phosphate in catalysis.

The mechanism proposed requires stabilization of the activated glucosyl moiety. X-ray crystallography has shown that there are no charged amino acid side chains at the back side (*re* side; above the plane) and no negatively charged groups in the vicinity of O-5 and C-1 of the sugar. This leaves the anionic substrate phosphate at the front side as the best candidate for the stabilization and the subsequent nucleophilic attack on C_1 of the glucosyl residue (Klein et al., 1984a, 1986; McLaughlin et al., 1984; Hajdu et al., 1987; Palm et al., 1987b). Stabilization of the intermediate transition-state-like complex from the *si* side by the substrate phosphate would at the same time prevent the phosphate from diffusing away and would be compatible with the exchange of the ester and phosphoryl oxygens of α-D-glucose 1-phosphate (Kokesh & Kakuda, 1977).

The proposed mechanism is a concerted mechanism with front-side attack (see Figure 5). This mechanism is a special case of a more general treatment of phosphorylases as glucosyl transferases. It was already anticipated by Mildred Cohn in 1961 as she wrote: "The most likely mechanism consistent with the experimental data for polysaccharide phosphorylase is a single displacement reaction with a front-side attack by the phosphate anion on carbon 1 of the terminal glucose". In a mechanism of this kind the functioning of the 5′-phosphate group of pyridoxal 5′-phosphate as a general acid (PL–OPO_3H^- or PL–OPO_3^{2-}) that transfers its proton to a substrate phosphate (or arsenate) can readily be imagined.

X-ray crystallographic and kinetic data and stereochemical considerations all suggest that the phosphorylase mechanism does not require distortion of the terminal sugar of the saccharide or of the glucosyl residue of glucose 1-phosphate prior to bond breaking. Barford et al. (1988) have pointed out that C–OR cleavage is unfavorable in the ground-state conformation of β-D-glycosides. In this case the sugar must be forced into a half-chair or sofa conformation in order to make the reaction possible. This is the case with lysozyme which handles

β-D-glycosides and where the sofa conformation of the glucosyl residue is preferred (Phillips, 1967; Imoto et al., 1972; Ford et al., 1974). On the other hand, in the case of α-D-glycosides, C–OR bond cleavage is stereoelectronically favorable in the ground-state conformation. This is the case for the phosphorylase reaction. Moreover, it should be recalled that in the phosphorylase reaction the phosphate stabilizes the glucosyl oxocarbonium ion intermediate. Accordingly, there is no need for the glucopyranose ring to be distorted. The role of the phosphate is emphasized by the consequences of mutations of charged amino acids (e.g., Lys 568, Lys 574, and Glu 672).

The mechanism proposed is compatible with all the X-ray structural evidence available at present. Recently, additional support came from the analysis of crystals of rabbit muscle phosphorylase *b* tetramers in the R state in the presence of sulfate and phosphate ions (Barford & Johnson, 1989). These new data reveal a close relationship of the active-site conformation of the R-state phosphorylase with that of the heptulose 2-phosphate–phosphorylase complex [cf. Hajdu et al. (1987)] on which our mechanistic interpretation rests.

A Rationale for a Catalytic Role of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase

It was previously pointed out (Klein & Helmreich, 1985; Palm & Schinzel, 1989) that the important evolutionary advantage gained on the emergence of phosphorylases was associated with their capability of cleaving "energy-rich" glucose polymers phosphorolytically rather than hydrolytically like the majority of polymer-degrading enzymes. Phosphorolysis preserves the energy of the glycosidic bond in sugar phosphate esters which then can generate energy in subsequent glycolytic reactions. This requires an efficient way of excluding water from the catalytic site. That this is realized in the case of phosphorylase has now been shown by site-directed mutagenesis which indicated that Tyr 573, which is hydrogen-bonded to the 2-OH of the glucosyl residue, assists in the exclusion of water because its substitution by Phe increased the ratio of hydrolysis to phosphorolysis. We would like to speculate that the preference of α-glucan phosphorylases for phosphorolysis rather than hydrolysis is related to a novel catalytic feature that these enzymes have acquired and that has enabled them to take advantage of a "mobile phosphate anion" receiving protons from the phosphate of the cofactor. Thus, in phosphorylases, a pair of phosphates, which directly interact, has replaced the pair of amino acid carboxyl groups in carbohydrases. One of the reasons why nature has made use of a vitamin B_6 analogue in phosphorylase catalysis is probably that no amino acid side chain besides histidine could accomplish an equally well balanced proton transfer. In addition, pyridoxal 5′-phosphate has a different positional mobility than do amino acid side chains constrained by protein structure. An intriguing question is whether these features of the catalytic mechanism facilitated the selection of the extremely versatile means of activity control by promoting substrate and effector binding (Sprang et al., 1988; Barford & Johnson, 1989). But all what one can say at present is that in a general way it appears that the multiplicity of phosphate binding sites both in the catalytic and in the regulatory domain is a characteristic feature in the evolution of regulatory enzymes. This applies to muscle phosphorylase as well as to phosphofructokinase (Poorman et al., 1984).

Conclusions

A mechanism for the phosphorylase reaction is proposed: In the forward direction, e.g., phosphorolysis of α-1,4-glycosidic bonds in oligo- or polysaccharides, the reaction is started by protonation of the glycosidic oxygen by orthophosphate, followed by stabilization of the incipient oxocarbonium ion by the phosphate anion and subsequent covalent binding of the phosphate to form α-glucose 1-phosphate. In the reverse direction, protonation of the phosphate of glucose 1-phosphate destabilizes the glycosidic bond and promotes formation of a glucosyl oxocarbonium ion–phosphate anion pair. In the subsequent step the phosphate anion becomes essential for promotion of the nucleophilic attack of a terminal glucosyl residue on the carbonium ion. This sequence of reactions brings about α-1,4-glycosidic bond formation and primer elongation. This mechanism accounts for retention of configuration in both directions without requiring sequential double inversion of configuration. It also provides for a plausible explanation of the essential role of pyridoxal 5′-phosphate in glycogen phosphorylase catalysis: The phosphate of the cofactor, pyridoxal 5′-phosphate, and the substrate phosphates approach each other within a hydrogen-bond distance allowing proton transfer and making the phosphate of pyridoxal 5′-phosphate into a proton shuttle which recharges the substrate phosphate anion.

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Our efforts to interpret the catalytic mechanism of α-glucan phosphorylases and the role of pyridoxal 5′-phosphate in catalysis profited greatly from productive and harmonious cooperation and exchange of ideas between the Oxford group and our group. With respect to this paper, we are especially indebted to Dr. Louise N. Johnson and Dr. David Barford and their colleagues from the Laboratory of Molecular Biophysics, The University of Oxford, Oxford, U.K., for making available unpublished data on the 3-D structure of phosphorylase *b* and modeling of the active site (Figures 3 and 4). We are grateful to C. Möller for secreterial help.

Registry No. Pyridoxal 5′-phosphate, 54-47-7; glycogen phosphorylase, 9035-74-9.

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Accelerated Publications

In Vitro Mutagenesis Studies at the Arginine Residues of Adenylate Kinase. A Revised Binding Site for AMP in the X-ray-Deduced Model†

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ABSTRACT: Although X-ray crystallographic and NMR studies have been made on the adenylate kinases, the substrate-binding sites are not unequivocally established. In an attempt to shed light on the binding sites for $MgATP^{2-}$ and for AMP^{2-} in human cytosolic adenylate kinase (EC 2.7.4.3, hAK1), we have investigated the enzymic effects of replacement of the arginine residues (R44, R132, R138, and R149), which had been assumed by Pai et al. [Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37–45] to interact with the phosphoryl groups of AMP^{2-} and $MgATP^{2-}$. With use of the site-directed mutagenesis method, point mutations were made in the artificial gene for hAK1 [Kim, H. J., Nishikawa, S., Tanaka, T., Uesugi, S., Takenaka, H., Hamada, M., & Kuby, S. A. (1989) *Protein Eng. 2*, 379–386] to replace these arginine residues with alanyl residues and yield the mutants R44A hAK1, R132A hAK1, R138A hAK1, and R149A hAK1. The resulting large increases in the $K_{m,app}$ values for AMP^{2-} of the mutant enzymes, the relatively small increases in the $K_{m,app}$ values for $MgATP^{2-}$, and the fact that the R132A, R138A, and R149A mutant enzymes proved to be very poor catalysts are consistent with the idea that the assigned substrate binding sites of Pai et al. (1977) have been reversed and that their ATP-binding site may be assigned as the AMP site.

Adenylate kinase (EC 2.7.4.3, AK), a small and ubiquitous enzyme, catalyzes a phosphoryl-transfer reaction between $MgATP^{2-}$ (or $MgADP^{-}$) and uncomplexed AMP^{2-} (or uncomplexed ADP^{3-}). This enzyme has two distinct nucleotide substrate binding sites: a highly specific one for AMP and a less specific site for MgATP (Noda, 1973). Although three-dimensional X-ray diffraction measurements of AK [from porcine muscle (Sachsenheimer & Schulz, 1977; Dreusicke et al., 1988) and from yeast (Egner et al., 1987)] have been conducted, the substrate-binding sites are still a controversial issue. The substrate-binding sites, suggested by X-ray diffraction studies (Pai et al., 1977; Egner et al., 1987; Dreusicke et al., 1988), by NMR analysis [Fry et al., 1985, 1987; cf. Hamada et al. (1979) and Kuby et al. (1989)], and by molecular mechanics calculations (Caldwell & Kollman, 1988), differ from one another. The proposed binding sites from these studies do not satisfactorily provide a common nucleotide binding sequence motif, especially in the glycine-rich flexible loop region, which has been proposed as a sequence diagnostic of the nucleotide phosphate binding site (Möller & Amons, 1985; Fry et al., 1986). However, AK is often cited as an example of a typical ATP-binding protein when structural topological comparisons or amino acid homology comparisons are made of the nucleotide-binding proteins (Bradley et al., 1987; Garboczi et al., 1988; Taylor & Green, 1989).

Recently, we chemically synthesized the gene for the human cytosolic adenylate kinase (hAK1), expressed it in *Escherichia coli*, and demonstrated that Tyr-95 and Arg-97 were not directly involved in the binding of MgATP, from kinetic analysis of artificial mutant enzymes at these positions (Kim et al., 1989a). In this paper, we tested the X-ray-deduced model of Pai et al. (1977), by point mutation effects on apparent kinetic parameters of replacement of those arginine residues that are inclined toward the so-called "active center cleft" [as designated by Pai et al. (1977)]. These arginine residues appear to be conserved in the AK family [AK1, AK2, AK3, AKy, and AKe, as denoted by Schulz et al. (1986)], and they were replaced with alanyl residues (R44A, R132A, R138A, and R149A). The roles played by these arginine residues, which have been interpreted from their apparent kinetic parameters, made the original model of Pai et al. (1977) untenable, unless their binding site of AMP were interchanged with their ATP site.

Incidentally, a cursory examination was made of the role played by Asp-119. This residue was postulated by Fry et al. (1985, 1986), through an analogy to homologous systems

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