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ARTICLE

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Proton relay system in the active site of maltodextrinphosphorylase via hydrogen bonds with large proton polarizability: an FT-IR difference spectroscopy study

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Abstract Maltodextrinphosphorylase (MDP) was studied in the pH range 5.4–8.4 by Fourier transform infrared (FT-IR) spectroscopy. The pK_a value of the cofactor pyridoxalphosphate (PLP) was found between 6.5 and 7.0, which closely resembles the second pK_a of free PLP. FT-IR difference spectra of the binary complex of MDP + α-D-glucose-1-methylenephosphonate (Glc-1-MeP) minus native MDP were taken at pH 6.9. Following binary complex formation, two Lys residues, tentatively assigned to the active site residues Lys533 and Lys539, became deprotonated, and PLP as well as a carboxyl group, most likely of Glu637, protonated. A system of hydrogen bonds which shows large proton polarizability due to collective proton tunneling was observed connecting Lys533, PLP, and Glc-1-MeP. A comparison with model systems shows, furthermore, that this hydrogen bonded chain is highly sensitive to local electrical fields and specific interactions, respectively. In the binary complex the proton limiting structure with by far the highest probability is the one in which Glc-1-MeP is singly protonated. In a second hydrogen bonded chain the proton of Lys539 is shifted to Glu637. In the binary complex the proton remains located at Glu637. In the ternary complex composed of phosphorylase, glucose-1-phosphate (Glc-1-P), and the nonreducing end of a polysaccharide chain (primer), a second proton may be shifted to the phosphate group of Glc-1-P. In the doubly protonated phosphate group the loss of mesomeric stabilization of the phosphate ester makes the C_1-O_1 bond of Glc-1-P susceptible to bond cleavage. The arising glucosyl carbonium ion will be a substrate for nucleophilic attack by the nonreducing terminal glucose residue of the polysaccharide chain.

Key words Maltodextrinphosphorylase · Hydrogen bonds · Proton polarizability · Fourier transform infrared spectroscopy · Pyridoxalphosphate dissociation state

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Introduction

α-Glucan phosphorylases play a key role in carbohydrate metabolism. They catalyze the phosphorolytic breakdown of glycogen and other storage polysaccharides, forming α-D-glucose-1-phosphate (Glc-1-P) with retention of configuration. The reaction is reversible in vitro. Structure and basic functions of phosphorylases have been highly conserved throughout evolution (Newgard et al. 1989; Hudson et al. 1993). All α-glucan polysaccharide phosphorylases known so far require pyridoxal-5′-phosphate (PLP) as a cofactor, which is covalently bound by a Schiff base to a lysine residue in the enzyme. To study the mechanism of phosphorylases the interest has been primarily focused on the role of PLP. Earlier studies (Fischer et al. 1958) have shown that the Schiff base could be reduced without loss of activity. Hence the function of the cofactor is different from all other vitamin B_6-dependent enzymes. Studies (Helmreich and Klein 1980) with pyridoxal-5′-phosphate analogs showed that only those analogs that contribute a reversibly protonatable dianion in the 5′-position were able to reconstitute an active enzyme. Furthermore, Parrish and coworkers (Parrish et al. 1977) could show that apophosphorylase reconstituted with pyridoxal exhibited activity only in the presence of orthophosphate or phosphate analogs. Madsen, Fukui and their colleagues proposed (reviewed in Madsen and Withers 1986), on the basis of noncovalent interactions between the 5′-phosphate group of the cofactor and the phosphate group of Glc-1-P, a role of PLP in the phosphorylase reaction where a “constrained”

phosphate dianion functions as an electrophilic catalyst which on attack of Glc-1-P would labilize the glycosidic bond in Glc-1-P. One reason to consider this mechanism unlikely comes from X-ray crystallography (Johnson 1992), showing that in rabbit muscle phosphorylase the atomic positions are too distant to allow direct phosphorus-phosphate contacts. This is also confirmed by the X-ray analysis of the maltodextrinphosphorylase (MDP) of *Escherichia coli* itself (Watson et al. 1997). Alternatively, a mechanism was proposed whereby the phosphate group of the cofactor participates in promoting the protonation of the substrate phosphate, which leads to destabilization of the glycosidic bond (Klein et al. 1982; reviewed in Palm et al. 1990).

To obtain further insight into this mechanism, it would be of importance to know the dissociation state of the cofactor and substrate phosphates in the unliganded enzyme and in the course of the reaction. In the past, use has been made of phosphorus NMR to probe the dissociation state of the cofactor phosphate. Despite the fact that the interpretation of ^{31}P NMR spectra is largely empirical, and the pH dependence of the ^{31}P chemical shift does not necessarily indicate protonation of the cofactor phosphate itself but may reflect the protonation of nearby residues in the protein (Gorenstein 1984; Porcuban et al. 1979), it could be shown that changes in phosphorylase activity are always running in parallel with changes of the chemical shift, suggestive of changes in the protonation state of the phosphate group (Palm et al. 1979; Schinzel et al. 1992).

To establish a proton donor-acceptor shuttle between distant phosphate groups it was suggested that an extended system of hydrogen bonds could make up a proton relay system (Johnson et al. 1992). In such a system, protons can be shifted via hydrogen bonds with large proton polarizability due to collective tunneling of the protons (Eckert and Zundel 1988a, b; Zundel 1992a).

It was previously found that hydrogen bonds between phosphate groups may show large proton polarizability (Schiöberg et al. 1974; Matthies and Zundel 1977). In addition, hydrogen bonds and hydrogen bonded systems with lysine, tyrosine, or glutamic acid and phosphate groups also show this property (Burget and Zundel 1986, 1987, 1988). Proton polarizabilities of hydrogen-bonded systems are indicated in the IR spectra by continuous absorptions (Zundel 1976, Zundel 1992b).

In the present work, Fourier transform infrared (FT-IR) spectroscopy was used to provide independent evidence of the dissociation state of the cofactor phosphate in phosphorylases, as well as the dissociation state of Lys and Glu residues in the active center of MDP with and without substrates. The different dissociation states of the groups mentioned above give rise to different bands in the FT-IR spectra. Hence, FT-IR spectroscopy seems to be well suited to probe the dissociation state of the phosphate group of the cofactor and of Lys and Glu residues. The use of difference spectra of wild-type and mutant enzyme should further allow us to determine the protonation state of individual active site residues in the native enzyme and in the binary complex. Thus, the percentage of the proton transfer in the easily polarizable $OH \cdots {}^{-}O \leftrightharpoons O^{-} \cdots OH$ and the $NH \cdots {}^{-}O \leftrightharpoons N^{+} \cdots HO$ bonds can be determined between phosphates, Lys and phosphates, and Lys and Glu, respectively.

Materials and methods

PLP was purchased from Sigma in spectroscopic grade and used without further purification. Glucose-1-methylenephosphonate (Glc-1-MeP) was prepared according to the procedure given by Becker et al. (1995).

Enzyme purification and sample preparation

The MDP of *E. coli* was overexpressed from the cloned *mal*P gene and purified according to the procedure given by Schinzel et al. (1992). Site directed mutagenesis was accomplished by the gapped duplex approach (Kramer and Fritz 1987) as described by Schinzel and Palm (1990). Two milligrams of the enzyme were desalted and concentrated to about 50 µl in a centrifugal concentrator and rediluted in 1 ml bidestilled water. For further purification the enzyme solution was dialyzed in a 10^{-3} M NaCl solution overnight at the respective pH values. The activity was tested according to Schinzel and Palm (1990). The binary complex was obtained by mixing the MDP with Glc-1-MeP in a molar ratio of 1 : 5.

IR spectroscopy

The spectra were recorded with a Bruker IFS 113v FT-IR spectrometer with a resolution of 2 cm^{-1} using a MCT detector. Attenuated total reflection (ATR) spectra were taken with an internal reflection element. A germanium crystal 52×18×2 mm with an aperture angle of 45° was used. Either 100 µl of the protein solution or 100 µl of the solution of the binary complex with a concentration of 2 mg/ml were spread and dried on this internal reflection element under a stream of dry nitrogen gas. The pH of the films in the range of interest between 5.4 and 8.4 was found to be stable compared with the pH of the solutions. This was checked for films of various proteins by using internal standards. Then the sample was rehydrated at 75% relative humidity of the nitrogen gas for several hours in the spectroscopic cell until the sample was equilibrated. Equilibrium in hydration was checked by taking control spectra every 30 min. These control spectra were subtracted from each other and the equilibrium was reached when no detectable difference in the water content was observed. The 75% relative humidity was obtained by conducting the dry gas very slowly through a solution of saturated sodium chloride in water (Stokes and Robinson 1949). The water content of the sample during the rehydration procedure was calculated by using the absorbance of the O–H stretching vibration of water at 3450 cm^{-1}. Absorptions caused by excess

of the substrate were avoided by subtracting a standard spectrum of the substrate phosphate (Glc-1-MeP) from the spectrum of the binary complex.

Difference spectra

In the case of the phosphorylase it was necessary to take difference spectra of two films. In this case, one has to pay high attention to avoid possible artifacts. For this requirement a self-made spectroscopic cell, based on a Perkin Elmer accessory, was constructed. This cell allows us to position the sample very exactly in the IR beam and this led to a very good reproducibility of the difference spectra of the two films. Nevertheless, one has to be aware of the fact that the intensities of the bands in the spectra can only be treated qualitatively.

The accuracy of the substraction of the excess of Glc-1-MeP was each time controlled by an external standard spectrum to make sure that the bands interpreted were not due to residual Glc-1-MeP.

Spectra were collected by taking 5×256 scans; then the average of these spectra were taken. The difference spectra shown are the averages of several cycles.

Results and discussion

pH-dependent changes in the FT-IR difference spectra of MDP

To study protonation and deprotonation of the functional groups at the active site of MDP, FT-IR difference spectra were taken from samples at different pH values. Figure 1a and Fig. 1b show the difference spectrum of MDP at pH 5.4 minus MDP at pH 8.4. In Fig. 1a a broad positive absorption in the region 3200–2500 cm^{-1} was found. It is caused by the $\nu(NH_3^+)$ vibration of the lysine residues in the protein, and implies that these residues are protonated at pH 5.4. A negative band was found in the region 3600–3200 cm^{-1} which is caused by $\nu(NH_2)$ of the lysine residues. Hence, these residues are deprotonated at pH 8.4.

In Fig. 1b, positive bands were found at 1202 cm^{-1} [$\nu_{as}(PO_2^-)$] and at 1090 cm^{-1} [$\nu_s(PO_2^-)$]. These vibrations are ascribed to the $(PO_2OH)^-$ group. Negative bands were observed at 1111 cm^{-1} [$\nu_{as}(PO_3^{2-})$] and at 975 cm^{-1} [$\nu_s(PO_3^{2-})$]. The changes observed with increasing pH demonstrate that the phosphate group of bound PLP becomes deprotonated in the pH range between 5.4 and 8.4. The band positions of the phosphate group of PLP are in accordance with spectra of PLP bound to aminotransferases (Sanchez-Ruiz and Martinez-Carrion 1986) as well as to other organic phosphates (Kempf and Zundel 1992). The band positions of PLP in the MDP fit also nicely to a recent FT-IR study of PLP itself (Bartl et al. 1998).

In order to get closer to the pK_a value of the phosphate group of the PLP, three further pH difference spectra were recorded (Fig. 2). The FT-IR difference spectrum of the sample at pH 6.5 minus 7.0 shows that the $\nu_s(PO_2^-)$ and $\nu_s(PO_3^{2-})$ bands appear with comparable intensities. In contrast, for other FT-IR difference spectra (pH 6.0–6.5 and pH 7.0–7.5, Fig. 2) no pH-dependent changes can be observed. Accordingly, the transition of the monoanionic to the dianionic form of the phosphate group of PLP takes place between pH 6.5 and 7.

The pH optimum of the activity of MDP was found close to 6.8 (Palm et al. 1976). The correspondence between the apparent pK of the cofactor phosphate and the pH dependence of the activity strongly suggests that the activity of

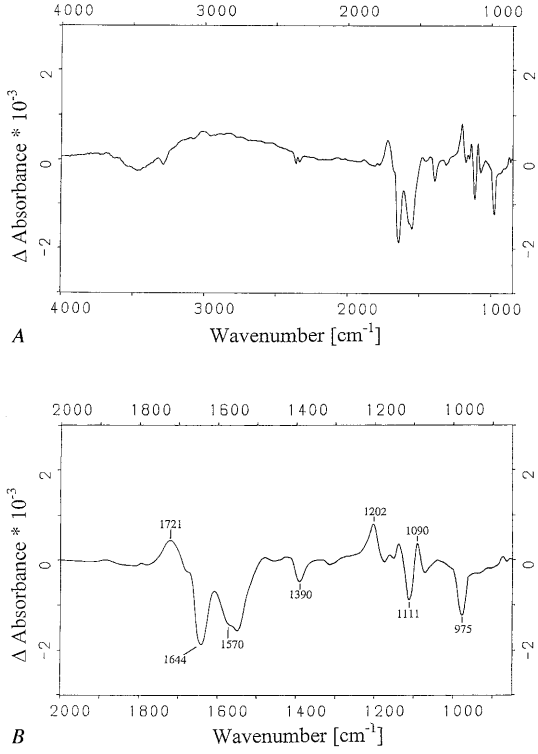


Fig. 1A, B FT-IR difference spectra of a hydrated film of maltodextrinphosphorylase (MDP): **A** pH 5.4 minus pH 8.4 in the region 4000–800 cm^{-1}; **B** pH 5.4 minus pH 8.4 in the region 2000–800 cm^{-1} (expanded part of **A**)

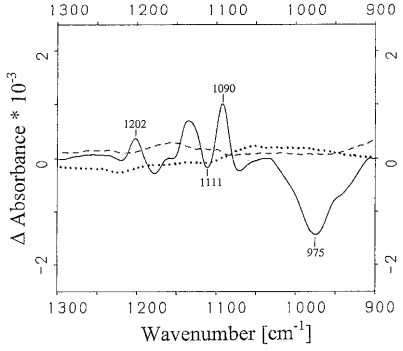


Fig. 2 FT-IR difference spectra of a hydrated film of MDP in the region 1300–900 cm^{-1}: (- - -), pH 6.0 minus pH 6.5; (——), pH 6.5 minus pH 7.0; (· · ·), pH 7.0 minus pH 7.5

the enzmye depends on the ability of the cofactor PLP to change its protonation state in the course of the reaction. At this pH the proton of the phosphate group can be transferred very easily, since the POH $\cdots$ $^{-}$OP $\leftrightharpoons$ PO^{-} $\cdots$ HOP phosphate-phosphate bonds show large proton polarizability.

These observations are in a marked contrast to the findings for another class of PLP-dependent enzymes. As shown by Sanchez-Ruiz and Martinez-Carrion (1986), in aminotransferases, no change of the dissociation state of PLP could be observed using FT-IR spectroscopy in a pH range from 4 to 9. In aminotransferases the aldehyde group of the cofactor is the functional group, forming a reversible Schiff base with the amino groups of their substrates. This and other evidence suggests that in aminotransferases the phosphate group most likely serves as an anchor for the cofactor but is not involved in catalysis (for review, see Jansonius and Vincent 1987). Therefore, the response of the cofactor phosphate group to pH changes in phosphorylases strongly supports a mechanism involving protonation/deprotonation as required by an acid/base mechanism (Palm et al. 1990).

In Fig. 1b a positive band at 1721 cm^{-1} [ν(C=O)] and negative bands at 1570 cm^{-1} [ν_{as} (CO_2^-)] and at 1390 cm^{-1} [ν_s (CO_2^-)] were found. These pH-dependent changes suggest that the carboxylic groups are deprotonated within this pH range. The negative bands at 1644 cm^{-1} (amide I band) and at 1551 cm^{-1} (amide II band) indicate that a small portion of α-helical structure is formed with increasing pH (Bellamy 1975).

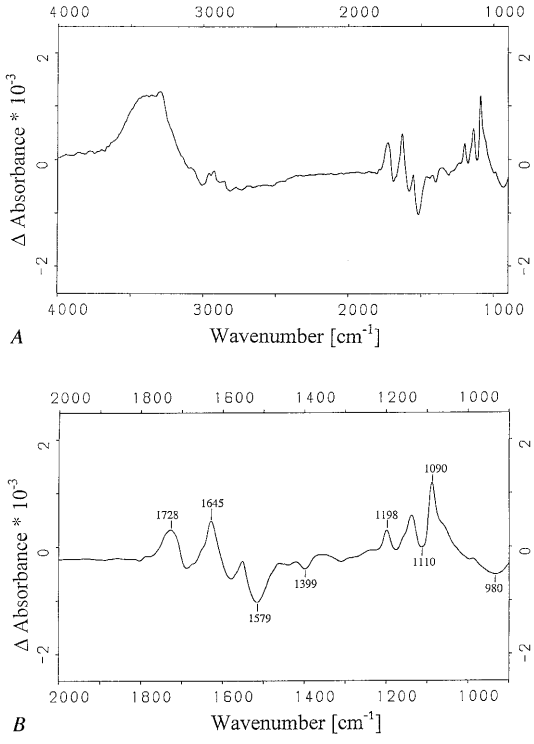


Fig. 3A, B FT-IR difference spectrum of a hydrated film of [MDP + glucose-1-methylenphosphonate (Glc-1-MeP)] minus [MDP]: **A** in the region 4000–800 cm^{-1}; **B** in the region 2000–800 cm^{-1} (expanded part of **A**)

The binary complex of MDP with Glc-1-MeP

Glc-1-MeP, which is one of the best competitive inhibitors of phosphorylases (Becker et al. 1995), was used to form a binary enzyme-substrate phosphate analog complex. The use of Glc-1-MeP instead of Glc-1-P allows us to keep the excess of ligand low.

In Fig. 3a the FT-IR difference spectra of MDP + Glc-1-MeP minus MDP are given. This figure shows a very broad negative $\nu(NH_3^+)$ band in the region 3200–2500 cm^{-1}. A new positive band with maximum at about 3300 cm^{-1} is observed. The disappearance the $\nu(NH_3^+)$ band following the addition of Glc-1-MeP implies that the lysine residues in the active site of the MDP become deprotonated due to the presence of Glc-1-MeP. The positive band with a maximum at about 3300 cm^{-1} is caused by the $\nu(NH_2)$ vibration of these lysine residues. The weak positive band at 3250 cm^{-1} is caused by the ν(OH) vibration of the glycosyl group of residual free Glc-1-MeP.

Figure 3b shows the respective difference spectra in the region 2000–900 cm^{-1}. A positive band at 1728 cm^{-1} [ν(C=O)] and negative bands at 1579 cm^{-1} [ν_{as} (CO_2^-)] and at 1399 cm^{-1} [ν_s (CO_2^-)] indicate that a carboxyl group in the active center is protonated upon formation of the MDP-Glc-1-MeP complex. The side chain of Glu637 is the only carboxyl group at the active site and therefore it is likely that the latter became protonated upon formation of the binary complex. To confirm this, similar measurements were performed with a mutant phosphorylase in which Glu637 was exchanged for a glutamine residue.

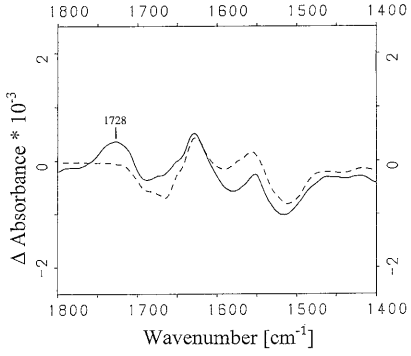


Fig. 4 FT-IR difference spectrum in the region 1800–1400 cm^{-1} of a hydrated film of: (——), [MDP (wild-type) + Glc-1-MeP] minus [MDP (wild-type)] at pH 6.5; (- - -), [MDP (E $\rightarrow$ Q) mutant enzyme + Glc-1-MeP] minus [MDP (E $\rightarrow$ Q) mutant enzyme], at pH 6.5

Figure 4 shows the difference spectrum of wild-type MDP complexed with Glc-1-MeP minus unliganded wild-type MDP (solid line) and the FT-IR difference spectrum of E637Q mutant enzyme complexed with Glc-1-MeP minus E637Q mutant enzyme (dashed line). The comparison of the two FT-IR difference spectra shows that only in difference spectra of the wild-type MDP is the ν(C=O) band at 1728

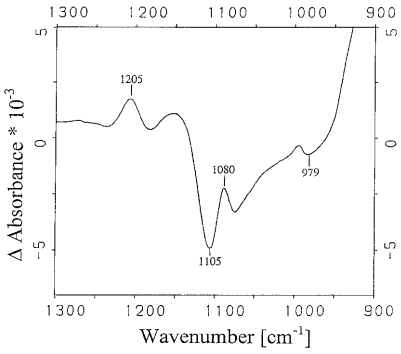


Fig. 5 FT-IR difference spectrum of [MDP]+dihydrogenarsenate] minus [MDP], in the region 1300–850 cm^{-1} at pH 6.5

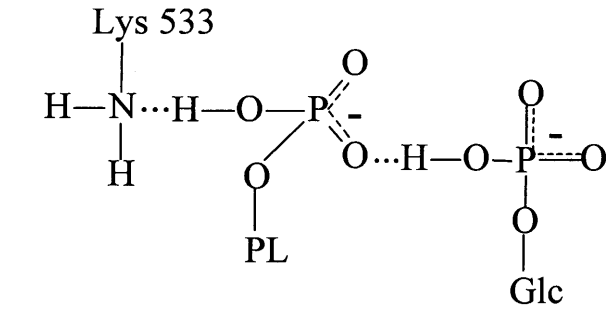
cm^{-1} observed. Thus, as assumed above, this band is due to the protonated Glu637 residue. A transfer of the proton from Lys539 to Glu637 along the ε-amino-lysyl to γ-carboxy-glutamyl hydrogen bond might account for this protonation. Furthermore, Fig. 3b shows positive bands at 1198 cm^{-1} [ν_{as} (PO_2^-)] and at 1090 cm^{-1} [ν_s (PO_2^-)] and negative bands at 1110 cm^{-1} [ν_{as} (PO_3^{2-})] and at 980 cm^{-1} [ν_s (PO_3^{2-})], respectively. These bands indicate that phosphate groups are protonated with addition of the substrate phosphate.

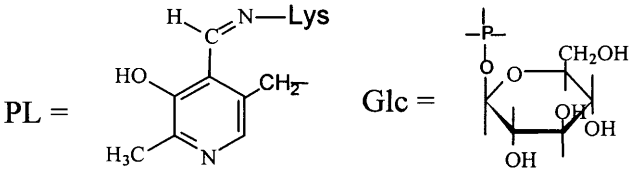
To distinguish between the phosphate group of the substrate phosphate and the phosphate groups of PLP, we used sodium arsenate as substrate analogon (Leuchs and Zundel 1979). Arsenate acts as a competitive inhibitor with respect to inorganic phosphate and shows no bands in the phosphate band region. The difference spectrum with the arsenate as substrate analogon in Fig. 5 shows almost the same positive and negative phosphate bands. This result supports the assumption that, with the addition of the substrate phosphate, the phosphate group of PLP becomes protonated while the protonation state of the substrate phosphate remains unchanged.

All these results, taken together with the X-ray structural data (Johnson 1992), suggest that in the binary complex the proton of Lys533 is present at the phosphate group of PLP and the proton of Lys539 is present at Glu637.

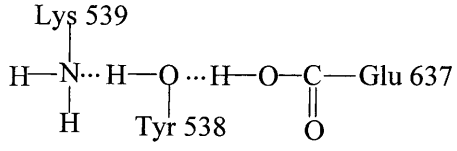
Now the question arises how the transfer of these protons can be realized:

1. The shift of the proton from Lys533 to PLP can be realized in a Lys-phosphate hydrogen bond. Lysine phosphate hydrogen bonds, i.e. $NH^+ \cdots {}^-OP \leftrightharpoons N \cdots HOP$ bonds, are bonds with large proton polarizability (Burget and Zundel 1986), in which the proton can easily be shifted by changes of the local electrical fields or by specific interactions. Furthermore, $POH \cdots O^-P \leftrightharpoons P^-O \cdots HOP$ hydrogen bonds are also easily polarizable (Schiöberg et al. 1974; Matthies and Zundel 1977). This hydrogen-bonded chain shown in Scheme 1 led us to expect large proton polarizability due to collective proton motion (Eckert and Zundel 1988a, b; Zundel 1992a, b). This result was recently confirmed by studies with model systems (Bartl et al. 1996). A chain built up by an amine, PLP, and glucose-1-hydrogenphosphate causes an intense continuum, demonstrating that such a chain shows large proton polarizability. In the binary complex according to the above-mentioned results, Lys533 is deprotonated, PLP and the substrate phosphate protonated. Thus this chain is strongly polarized by local electrical fields and specific interactions. Hence only the proton limiting structure shown in Scheme 1 is realized. In this way the proton becomes localized in the binary complex at the substrate phosphate.
2. Furthermore, the Glu637 residue is protonated by the Lys539 residue. This protonation occurs probably via the hydrogen bonded chain shown in Scheme 2. The proton remains located at the Glu637 residue in the binary complex. Thus, Lys539 cannot perform a second protonation of the substrate phosphate. Owing to such a protonation in the binary complex, the catalytic mechanism would be interrupted. How can the transfer of the proton from Lys539 to Glu637 be realized?





Scheme 1



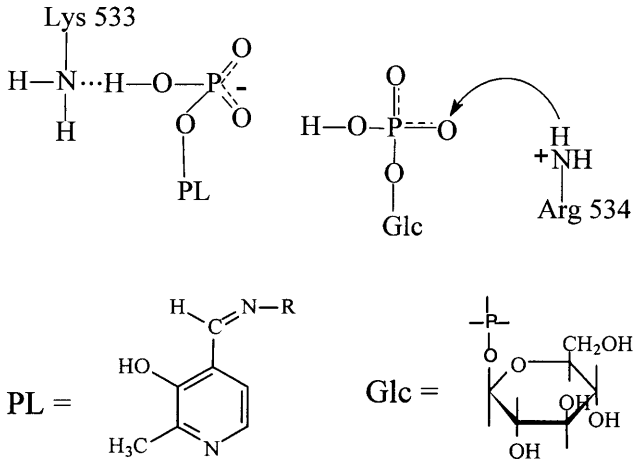
Scheme 2

The Lys-Glu hydrogen bonds are highly asymmetrical hydrogen bonds, with the deeper well at the lysine residue (Kristof and Zundel 1980). This result seems to contradict the result obtained with the enzyme-substrate complex. The X-ray data (Johnson 1992) suggest, however, that Tyr538 is present between Lys539 and Glu637. Hence, a chain may be assumed as shown in Scheme 2.

A hydrogen-bonded chain as shown in Scheme 2 is expected to be highly sensitive against local electrical fields

and specific interactions. This is particularly true since Lys-Tyr hydrogen bonds show large proton polarizability (Kristof and Zundel 1980). Thus the excess proton of the Lys539 may be shifted by the environment to Tyr538, and in a collective process the proton of Tyr538 could be shifted to Glu637 and remain located there. The studies with model systems (Bartl et al. 1996) confirm that the proton in such a chain is localized at a carboxylic group.

The shift of the excess proton from Lys539 to Glu637 as shown in Scheme 2 is essential for the mechanism to avoid the transfer of the proton to the phosphate group of the substrate phosphate, because this would lead to a doubly protonated phosphate group. The C_1–O_1 bond of the substrate phosphate would then be split, as discussed in the next section. This should be avoided in the binary complex in order not to interrupt the catalytic mechanism. Hence in the binary complex one proton is localized at the substrate phosphate and one at Glu637.



Scheme 3

A proposed model of functional binary and ternary complexes of MDP with Glc-1-P and the polysaccharide primer

The high concentrations of oligosaccharide required to achieve saturation at the active site make a determination of the protonation status of PLP in the ternary complex not feasible. In order to provide a basis to explain and understand the function of phosphorylases, it is necessary to extend the experimentally supported model of the binary complex to the ternary complexes constituted either by the enzyme, Glc-1-P, and polysaccharide or the enzyme, orthophosphate (P_i), and polysaccharide.

Analogous to the formation of the MDP-Glc-1-MeP complex, a binary MDP-Glc-1-P complex will be formed. Expecting further that the active-site structure will closely resemble the hydrogen-bonded systems illustrated in Schemes 1 and 2 with Glc-1-MeP, the limiting structure with a monoprotonated Glc-1-P has by far the largest weight. In aqueous solutions at pH = 7 the free enthalpy for the hydrolysis of free Glc-1-P is negative, i.e. this bond is thermodynamically not stable. In view of the fact that Glc-1-P is perfectly stable in the absence of the polysaccharide primer, the binary complex appears kinetically inhibited. Hence the loss of energy from mesomerism of the electronic system in the presence of one proton cannot be the only reason that allows Glc-1-P react to form more stable products.

To overcome the kinetic inhibition it is conceivable that one of the functions of the primer would be to modulate further the proton relay systems such as to allow for even more efficient destabilization of Glc-1-P. This would be possible if, on binding of the polysaccharide to the active site, the hydrogen bond between PLP and Glc-1-P in the binary complex is broken (Scheme 3). Now a second protonation occurs to destabilize the C_1–O_1 bond. This second protonation may occur by Arg534, which is also present in the active center (Johnson 1992).

Another possibility is that due to changes of the local fields and specific interactions with the formation of the ternary complex, the proton present at Glu637 returns to Lys539. The now-protonated Lys539 residue may perform the second protonation of the phosphate group of the substrate phosphate.

In the proposed doubly protonated structure, the former stabilization of the electronic system of the charged phosphate group by mesomeric effects has vanished completely. The loss of the mesomeric effect destabilizes the C_1–O_1 bond of Glc-1-P, resulting in cleavage of the natural substrate. The nascent glucosyloxocarbonium ion is stabilized by the negatively charged free phosphate. Ultimately the glucosyloxocarbonium ion will be subject to nucleophilic attack by the terminal glucose residue of the primer and, thus, adds a glucose unit for elongation of the polysaccharide chain.

At high concentration of free phosphate and a $K_{eq} = P_i/\text{Glc-1-P} = 3.6$ (Palm et al. 1990), phosphorolysis of polysaccharides will be favored. The steps describing the formation of the binary and ternary complexes in the direction of polysaccharide degradation are similar to those discussed in the direction of synthesis, with the difference that orthophosphate replaces Glc-1-P. The described hydrogen-bonded chain with large proton polarizability renders possible the microreversibility of the reaction.

Of course, these considerations are only speculations with regard to the ternary complex. They are, however, very well supported by the knowledge of the nature of the binary complex.

Conclusions

FT-IR spectroscopy seems to be well suited to probe the dissociation state of the phosphate group of PLP, as well as to determine the protonation state of individual active site residues. Concerning the protonation of phosphate groups, FT-IR has provided independent evidence for former assignments of the protonation state by ^{31}P NMR (Palm et al. 1990; Schinzel et al. 1992; Becker et al. 1995).

In the unliganded MDP the phosphate group of PLP is monanionic below pH 6.5 and dianionic above pH 7.0. In the binary complex formed with the substrate phosphate analog Glc-1-MeP and MDP, PLP and the carboxylate group of Glu637 are protonated. The ε-NH_3^+ groups of two lysines are deprotonated. With regard to the structure of the binary complex, the Lys534 proton has been transferred to PLP. Hence, a hydrogen-bonded chain as shown in Scheme 1 is present. This chain is easily polarizable due to collective proton motion. In the binary complex this chain is, however, so strongly polarized that the proton is localized at the substrate phosphate.

In the binary complex in the absence of a primer the limiting structure outlined in Scheme 1 is expected to have the largest weight. As far as it concerns the monoanionic form of Glc-1-P, the C_1–O_1 phosphate ester bond is stabilized by mesomerism and the cleavage of the C_1–O_1 bond is kinetically inhibited. On formation of the ternary complex in the presence of the polysaccharide primer, the hydrogen bond between Glc-1-P and PLP will be broken (Scheme 3). A second protonation of the phosphate group of the substrate phosphate may occur by the conserved Arg534 residue. Another possibility is that the proton present at Glu637 is shifted again in the chain, as shown in Scheme 2, to Lys539 by a change of the local electrical fields and specific interactions. This protonated Lys may now perform the second protonation of the substrate phosphate. The doubly protonated phosphate will no longer be stabilized by mesomerism (Scheme 3). In effect, the C_1–O_1 bond will be destabilized and split. A glucosyloxocarbonium ion is formed in this process and will be transiently stabilized by the phosphate anion.

The fate of the glucosyloxocarbonium ion will depend on the reaction conditions: in the synthetic mode it will be subject to nucleophilic attack by the nonreducing terminal glucose residue of the priming polysaccharide, and in the degradation mode it will react with the phosphate anion itself (Palm et al. 1990).

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