Pyridoxal 5′-Phosphate as a ^{31}P Reporter Observing Functional Changes in the Active Site of *Escherichia coli* Maltodextrin Phosphorylase after Site-Directed Mutagenesis†

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ABSTRACT: Changes in the active site of *Escherichia coli* maltodextrin phosphorylase created by substituting residues Lys533, Arg534, Tyr538, and Glu637 were monitored in the absence and presence of arsenate as substrate analogue using pyridoxal-P as ^{31}P NMR reporter. The chemical shift of the cofactor phosphate group of wild-type *E. coli* phosphorylase is pH dependent with an apparent p*K* of 5.6 and limiting δ values of 0.71 and 3.6 ppm for the low- and high-pH values, respectively. The apparent p*K* value of 5.6 indicates that the phosphate group of the cofactor is in hydrogen bond linkage to Lys533. In all mutant enzymes in which the enzymatic activity was significantly reduced, effects on the ^{31}P chemical shift pattern of pyridoxal-P were observed. The K533S, R534Q, E637D, and E637Q mutant enzymes show 0.6, 0.01, 0.2, or 0.1% residual activity, and the apparent p*K* values of the cofactor phosphate transition of E637D and E637Q mutant enzymes are altered. The Y538F mutant enzyme is a remarkable exception, displaying 12% activity and an environment of the cofactor quite similar to that in wild-type enzyme. This finding suggests that Tyr538, although involved in substrate binding and specificity, is not functionally essential. One crucial aspect of catalysis is the close contact of the phosphates of pyridoxal-P and of substrate rendered by a cluster of positively charged amino acids, Lys533, Lys539, and Arg534. The similar apparent p*K* values of wild-type and K533S mutant phosphorylase suggest that the cofactor phosphate and the hydroxyl group of Ser533 are linked by a hydrogen bond. Model building studies using the R-state of muscle phosphorylase predict that such a hydrogen bond distance can only be provided by a movement of the cofactor pyridine ring including a distortion of O–P–O bond angles of the phosphate group which in turn causes the observed reduction in total ^{31}P chemical shift. Since residues Arg534 and Glu637 are not in close contact to the cofactor phosphate, the interpretation of the ^{31}P NMR data must include the participation of other neighboring amino acid residues. The ^{31}P NMR spectroscopy implies differences in binary complexes of *E. coli* mutant phosphorylases with arsenate when compared to that of native enzyme.

Pyridoxal 5′-phosphate dependent α-glucan phosphorylases (EC 2.4.1.1) catalyze the phosphorolytic cleavage of storage polysaccharides, resulting in the release of glucose 1-phosphate. One of the early applications of ^{31}P nuclear magnetic resonance (NMR)[1] studies to establish the function of pyridoxal-P in a catalytic process was the determination of the ionization state of this cofactor (Feldmann & Hull, 1977). These studies made it apparent that the reversible ionization of the phosphate group of pyridoxal-P is essential for catalysis. Studies of phosphorylase apoenzyme reconstituted with pyridoxal-P analogues and of analogues of inorganic phosphate suggested further that close contacts of the cofactor phosphate with the substrate phosphate (P_i) are obligatory for catalysis [for recent reviews, see Madsen and Withers (1986); Johnson et al., (1990), and Palm et al. (1990)]. X-ray crystallographic studies on rabbit muscle phosphorylase *b* coupled with NMR studies and experiments with a new class of glycosylic substrate analogues led to the proposal of two alternative catalytic mechanisms for glycogen phosphorylase. While one mechanism counts on a constrained cofactor phosphate creating an electrophilic trigger for the attack on glucose 1-phosphate (Withers et al., 1982; Madsen & Withers, 1986), we proposed a mechanism which depends on the phosphate group of the cofactor as an acid–base catalyst promoting the protonation of the substrate phosphate (Klein et al., 1984; Palm et al., 1990).

The structural basis for understanding the catalytic mechanism was provided by in situ X-ray studies of product formation from heptenitol and orthophosphate in crystals of glycogen phosphorylase *b* (Hajdu et al., 1987; Johnson et al., 1990). Although the described mechanism is chemically plausible, the functional contribution of individual amino acid residues is still under discussion. Since the primary and proposed tertiary structures of domains involving substrate binding and catalytic action are almost identical among glycogen phosphorylases (Palm et al., 1985, 1986; Newgard et al., 1989), site-directed mutagenesis of maltodextrin phosphorylase was used to answer those questions. On the basis of the 3-D structure, a critical role of those amino acid side chains lining the catalytic site of maltodextrin phosphorylase proximal to the glucopyranosyl ring and the substrate phosphate, Glu637, Lys539, Tyr538, Arg534, and Lys533, was anticipated (Figure 1). The aim of this study is to establish a structure–function relationship of these amino acid side chains. Changes in the active site were monitored using the

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[1] Abbreviations: NMR, nuclear magnetic resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; pyridoxal-P, pyridoxal 5′-phosphate; δ, chemical shift; P_i, inorganic phosphate.

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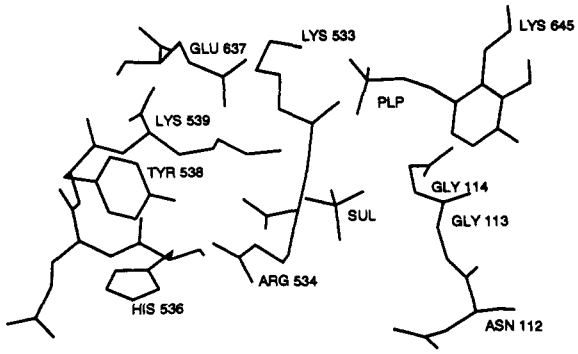

FIGURE 1: Active site of *E. coli* maltodextrin phosphorylase adapted from Barford and Johnson (1989) on the basis of the structure of rabbit muscle phosphorylase *b* in the R-state. The alignment of the appropriate amino acid residues from the sequences of *E. coli* maltodextrin and rabbit muscle phosphorylase is described by Schinzel and Palm (1990). SUL, sulfate residue.

cofactor phosphate group as a ^{31}P NMR reporter.

Materials and Methods

Enzyme Purification and Characterization. Construction of active-site mutants of maltodextrin phosphorylase and their kinetic characterization have been reported previously (Schinzel & Palm, 1990; Schinzel, 1991; Schinzel & Drueckes, 1991). Wild-type and mutant enzymes were purified as described (Schaechtele et al., 1978; Schinzel & Palm, 1990) with the following modifications. The ion-exchange chromatography was performed on a preparative FPLC Q-Sepharose column substituting for chromatography on DEAE-Sephacel. The Q-Sepharose column was equilibrated with 10 mM Tris–acetate/1 mM EDTA, pH 7.3, and the enzyme was eluted with a linear gradient of 0–0.3 M NaCl. The collected protein was dialyzed against 10 mM Tris–acetate/1 mM EDTA, pH 6.9. Purification of low-activity mutant protein was monitored by SDS–PAGE. The pyridoxal-P content was determined as described by Wada and Snell (1961). Protein was measured by the method of Bradford (1971) or obtained from the absorbance at 280 nm using $A^{0.1\%} = 1.36$.

Enzyme activity was assayed at 30 °C in the direction of phosphorolysis by measuring glucose 1-phosphate formation in a coupled assay whereas in the direction of synthesis the enzyme was tested by determining the release of P_i from glucose 1-phosphate (Schinzel & Palm, 1990). The time course of temperature inactivation was followed by incubating wild-type and mutant enzymes at 60 °C in 50 mM MES buffer, pH 6.5. Aliquots were drawn at different times, denatured protein was removed by centrifugation, and the residual activity was determined in the supernatant by the coupled assay described above. ELISA's were performed by applying a serial dilution of affinity-purified polyclonal antibody raised against maltodextrin phosphorylase and using a second antibody–horseradish peroxidase conjugate as indicator enzyme (Tijssen, 1988). The response of equal antibody dilution to 0.1 mg of wild-type or mutant enzyme/well was compared.

Sample Preparation. Ammonium sulfate precipitated maltodextrin phosphorylase was resuspended in 50 mM MES, 1 mM EDTA, 1 mM DTE, and 50 mM KCl and extensively dialyzed against the same buffer at the appropriate pH value. At pH values higher than 7.5, HEPES substituted for MES. The protein was concentrated to 10 mL using an AMICON 30 concentrator. The final protein concentration of the NMR samples varied from 150 to 200 μM. The pH of each sample was determined before and after the NMR experiment. After a series of NMR measurements, the enzymes were tested for loss of activity and cofactor content using the $A_{330/280}$ ratio (Schinzel & Palm, 1990).

Table I: Chemical Shift Data of Wild-Type and Mutant *E. coli* Phosphorylases

	apparent pK	chemical shift (ppm) at low pH	chemical shift (ppm) at high pH	Δ[a]	% activity
model Schiff base	6.2	1.05	4.17	3.12	
wild type	5.6	0.71	3.59	2.88	100
Y538F	5.3	0.25	3.64	3.39	12
K533S	5.5	1.74	3.06	1.32	0.6
E637D	6.4	1.67	2.8	1.21	0.2
E637Q	8.0	2.94	3.87	0.93	0.1
R534Q	5.8	2.19	3.36	1.17	0.01

[a] Total chemical shift.

NMR Techniques. Fourier transform ^{31}P NMR spectra were performed at 72.86 or 121.49 MHz on Bruker WH180 WB or AM300 SWB superconducting spectrometers. Enzyme samples were recorded in 20-mm-diameter tubes containing a concentric 5-mm NMR tube employed as field/frequency lock or in 10-mm-diameter tubes holding 10% deuterium oxide. All spectra were recorded with broadband proton decoupling (0.5 W). In general, a 1200-Hz spectral width was acquired in 4K data points with a 60° pulse angle and 1.7-s repetition time. For enzyme samples, the exponential line broadening used prior to Fourier transformation was usually 10 Hz. Continuous air flow through the spectrometer probe head kept the temperature at 20 ± 1 °C. A total of 20 000–30 000 transients were collected per experiment. The chemical shift scale is referenced to 85% H_3PO_4 as 0 ppm. Positive chemical shift values indicate resonances at fields lower than the reference. Transition curves were obtained by iterative computer analysis (Göbber & Lachmann, 1978; Lachmann & Schnackerz, 1984).

Results

Properties of Mutant Maltodextrin Phosphorylases. Mutant enzymes purified by affinity chromatography on glycogen–Sepharose columns elute at the same salt concentration as the wild-type enzyme. The cofactor content of all mutant enzymes was 1 mol of pyridoxal-P/mol of subunit. Identical $OD_{280/330}$ ratios indicate similar binding of pyridoxal-P into the cofactor binding site. The thermal stability of all mutant proteins tested was unaltered (data not shown). The response of affinity-purified anti-maltodextrin phosphorylase antibodies assayed at equal antibody dilutions was 100%. Therefore, it appears that the integrity of the overall structure is not altered by the described mutations.

Effect of pH on the ^{31}P Signal of Pyridoxal-P in Wild-Type and Mutant Enzymes. The pH dependence of the ^{31}P resonance of pyridoxal-P in wild-type and mutant maltodextrin phosphorylases is illustrated in Figure 2 and summarized in Table I. ^{31}P NMR spectra of wild-type enzyme show at pH 7.2 a single resonance with a line width of 30 Hz, similar to those reported by Palm et al. (1979). The ^{31}P signal of the phosphate group of pyridoxal-P is pH dependent with an apparent p*K* of 5.6 and limiting δ values of 0.71 and 3.59 ppm for the low- and high-pH values, respectively (Figure 2A, Table I). The replacement of Tyr538 by phenylalanine had a small effect on k_{cat}, lowering it to 12% of the value for wild-type enzyme. K_m values for substrates, P_i, glucose 1-phosphate, and maltoheptaose increased only slightly (Schinzel & Palm, 1990). The pH dependence of the ^{31}P chemical shift of the cofactor phosphate group exhibited a behavior similar to that of wild-type enzyme. An apparent p*K* value of 5.3 with lim-

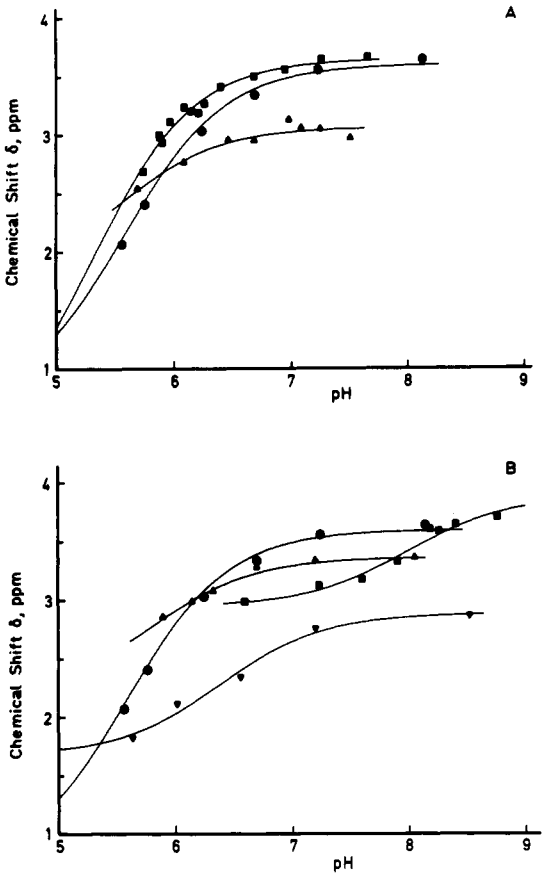

FIGURE 2: pH dependence of the ^{31}P chemical shift of pyridoxal-P bound to wild-type and mutant *E. coli* phosphorylases. (A) Wild-type enzyme (●); Y538F mutant enzyme (■); K533S mutant enzyme (▲). (B) Wild-type enzyme (●); E637D mutant enzyme (▼); E637Q mutant enzyme (■); R534Q mutant enzyme (▲). Experiments were performed in 50 mM HEPES, 50 mM KCl, 1 mM DTE, and 1 mM EDTA at 20 °C. For experimental details, see Materials and Methods. Transition curves were obtained by iterative computer analysis; Apparent p*K* values and chemical shift data are compiled in Table I.

iting δ values of 0.25 and 3.64 ppm was found for the transition of the cofactor phosphate group (Figure 2A). Lys533 is the only amino acid whose ε-amino group is in hydrogen bond distance to one of the oxygens of the cofactor phosphate. The K533S mutant enzyme exhibits 0.6% of the activity of the wild-type enzyme and shows a normal apparent p*K* of 5.5 for the transition of the cofactor phosphate group (Figure 2A). The total chemical shift difference, however, is less than half as large as that found for the wild-type enzyme (Table I). Addition of monovalent cations did not affect the ^{31}P NMR spectra of the K533S mutant enzyme although enzymatic activity can partially be restored by addition of monovalent cations (Schinzel, 1991). The E637D mutation results in an enzyme species which exhibits 0.2% of the activity of the wild-type enzyme whereas K_m values of substrates for phosphorolysis remain unchanged (Schinzel & Palm, 1990). The pH dependence of the ^{31}P chemical shift of the cofactor phosphate group is greatly affected when compared with the behavior of wild-type enzyme (Figure 2B). The apparent p*K* value is shifted by almost 1 unit to 6.4 with limiting δ values of 1.67 and 2.88 ppm for the low- and high-pH values, respectively. The effects are even more pronounced when Glu637 is substituted by the isosteric but uncharged glutamine residue. The E637Q mutant enzyme shows an activity of 0.1% of the wild-type enzyme. The affinities of substrates for this enzyme are similar to those of wild-type enzyme (Schinzel & Palm, 1990). The ^{31}P signal of the cofactor phosphate group

Table II: Chemical Shifts of Pyridoxal Phosphate in Wild-Type and Mutant *E. coli* Phosphorylases in the Presence of Arsenate at pH 7.2

	chemical shift (ppm)					
ligand	WT	Y538F	K533S	R534Q	E637D	E637Q
none	3.60	3.60	3.15	3.32	2.75	3.12
60 mM arsenate	3.37	3.38	2.94	3.10	3.90	1.63

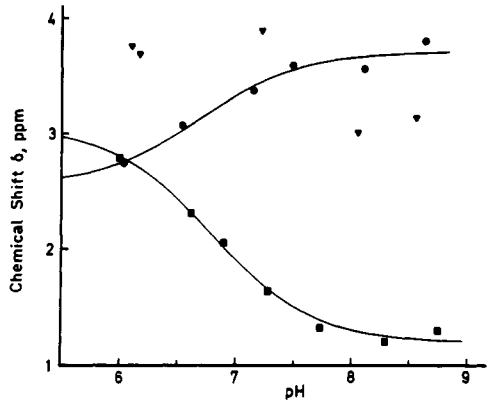

FIGURE 3: pH dependence of the ^{31}P chemical shift of pyridoxal-P bound to wild-type and mutant *E. coli* phosphorylases in the presence of 60 mM arsenate. Wild-type enzyme (●); E637Q mutant enzyme (■); E637D mutant enzyme (▼). Experiments were performed in 50 mM HEPES, 50 mM KCl, 1 mM DTE, and 1 mM EDTA at 20 °C. For experimental details, see Materials and Methods. Titration curves were obtained by iterative computer analysis. The apparent p*K* values are 6.7 and 6.8 for wild-type and E637Q mutant enzyme, respectively.

is pH dependent with an apparent p*K* of 8.0 and limiting δ values of 2.94 and 3.87 ppm for the observed transition (Figure 2B, Table I). Substitution of the charged Arg534 by a glutamine results in a 10^4-fold decrease in enzymatic activity whereas affinities of physiological substrates are not affected by this mutation. It has not yet unequivocally been proven that the residual activity of the R534Q mutant enzyme is not the result of contamination by small amounts of wild-type enzyme (Schinzel & Drueckes, 1991). The ^{31}P chemical shift of the phosphate group of pyridoxal-P in the R534Q mutant enzyme is pH dependent with an almost unchanged apparent p*K* of 5.8 and limiting δ values of 2.19 and 3.36 ppm for the low-pH and high-pH values, respectively (Figure 2B, Table I).

Effects of Arsenate on the ^{31}P Signal of Pyridoxal-P in Wild-Type and Mutant Enzymes. Maltodextrin phosphorylase can use arsenate as a slowly reacting substrate analogue or as a competitive inhibitor of orthophosphate. At pH 7.2, the ^{31}P chemical shifts of the cofactor phosphate of wild-type, Y538F, K533S, and R534Q mutant enzymes are quite similar in the presence and absence of 60 mM arsenate, respectively (Table II). However, when the addition of substrate analogue was followed over a wide pH range, the transition of the cofactor phosphate signal was different from that of unliganded native enzyme (Figure 3). An apparent p*K* value of 6.7 and limiting δ values of 2.54 and 3.74 ppm at low and high pH, respectively, were found for the cofactor transition. In contrast, the ^{31}P signal of the cofactor phosphate in unliganded E637D mutant enzyme is shifted in the presence of 60 mM arsenate downfield in the pH range 6.1–8.5 (Figure 3). Furthermore, the cofactor phosphate of the E637Q mutant enzyme reveals in the presence of 60 mM arsenate at pH 7.2 a large upfield shift to 1.6 ppm (Table II). When the pH is decreased, the phosphate signal of the E637Q mutant enzyme shows in the presence of 60 mM arsenate a downfield shift with an apparent p*K* of 6.8 (Figure 3). This apparent p*K* value is similar to that

of wild-type enzyme in the presence of 60 mM arsenate, but the direction of the transition is reversed.

Discussion

The reaction catalyzed by glycogen phosphorylases is crucially dependent on a pair of phosphate residues including the essential cofactor pyridoxal-P (Parrish et al., 1977). A direct involvement of either P_i or glucose 1-phosphate in promoting protonation or destabilization of the glycosylic bond emerged from kinetic and ^{31}P NMR experiments with both pyridoxal-P and glycosylic substrate analogues (Klein et al., 1982, 1984, 1986; Palm et al., 1990).

In contrast to other enzymes where ^{31}P NMR spectroscopy of proteins modified by site-directed mutagenesis was used to elucidate the binding of the cofactor (Birdsall et al., 1989; Marceau et al., 1989), *E. coli* maltodextrin phosphorylase provides a unique opportunity to approach its mechanism since the cofactor phosphate itself is directly involved in catalysis. Effective use of ^{31}P NMR requires an understanding of structural and environmental factors which influence the phosphorus chemical shifts. Theoretical approaches for the interpretation of ^{31}P chemical shifts for all phosphorus compounds have been attempted by several laboratories (Letcher & van Wazer, 1967; Purdela, 1971), but the explanation of ^{31}P NMR spectra is still largely empirical. Gorenstein (1975) has made the important observation that the ^{31}P chemical shift of phosphate esters correlates well with the smallest O–P–O bond angle. For a variety of alkyl phosphates, it could be shown that a decrease in the smallest O–P–O bond angle results in a deshielding of the phosphorus nucleus, providing an explanation for the unusual downfield shift upon ionization of an acyclic monoanion. The 3° reduction in the O–P–O bond angle of the phosphate dianion is consistent with a 4 ppm downfield shift. Charge alone is not responsible for the deshielding since the acyclic monoanion and free acid both have similar chemical shifts and similar O–P–O bond angles (Gorenstein, 1984). Comparing X-ray and ^{31}P NMR data of cytosolic aspartate aminotransferase, it could be shown that alterations in the ^{31}P chemical shift of 0.5 ppm result from a conformational change in the 5′-side chain, in which one of the structures, the protonated pyridoxal-P Schiff base, involves a near-eclipsed pair of bonds. Such a stressed conformation produces slight alterations in bond angles around the phosphorus which in turn cause the observed change in ^{31}P chemical shift (Schnackerz et al., 1989). With these results in mind, we will discuss changes in the ^{31}P chemical shift rather in terms of changes in the O–P–O bond angles than ionization. The apparent p*K* is used to indicate the midpoint of pH-dependent transitions of chemical shifts. 3-D structural data of rabbit muscle phosphorylase in the R-state (Barford & Johnson, 1989) and of the heptulose 2-phosphate complex (Johnson et al., 1990) will be employed for the interpretation in the ground state or the binary and ternary complexes of the enzyme, respectively.

In view of the amino acid residues forming the active site of maltodextrin phosphorylase, five amino acids are prospective candidates as active participants in catalysis or substrate binding (Figure 1). Three of them (Lys533, Arg534, and Lys539) provide an accumulation of positive charges proximal to the catalytic center in hydrogen bond distance to the phosphate groups of cofactor and substrate. Two negatively charged or polar residues (Glu637 and Tyr538) are able to form hydrogen bonds to the glucose residue and might be involved in binding and catalysis.

The differences in chemical shifts of the ^{31}P NMR signal of mutant phosphorylases could be a consequence of conformational rearrangements introduced by the described mutations. Biochemical and biophysical parameters of wild-type and mutant enzymes were compared to exclude gross structural perturbations. The integrity of the oligosaccharide substrate binding site appeared not to be perturbed since the apparent binding constant was not affected by the mutations (Schinzel & Palm, 1990; Schinzel, 1991; Schinzel & Drueckes, 1991).

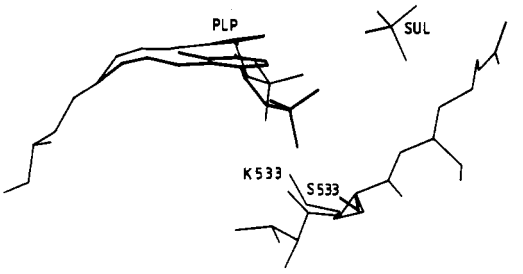

FIGURE 4: View of the cofactor phosphate hydrogen bonded to Lys533 in the active site and movement of the cofactor to facilitate a hydrogen bond of its phosphate group to the hydroxyl group of Ser533 (boldface).

For all mutant enzymes with significantly reduced enzymatic activity, effects on the ^{31}P chemical shift pattern of pyridoxal-P were observed. The apparent p*K* of the transition of the ^{31}P chemical shift of pyridoxal-P in wild-type *E. coli* phosphorylase is offset by 0.6 pH unit from 6.2 to 5.6 when compared to a model Schiff base of pyridoxal-P with ε-aminocaproate (Table I) (Palm et al., 1979). When this apparent p*K* shift is related to similar observations in D-serine dehydratase and other pyridoxal-P-dependent enzymes (Schnackerz et al., 1979; Schnackerz, 1984), it can be concluded that a negatively charged oxygen of the cofactor phosphate forms a salt bridge with a positive group in the active site. The 3-D structure of muscle phosphorylase in the R-state identified the positive charge as the ε-amino group of Lys568 (Lys533 in *E. coli* phosphorylase) (Barford & Johnson, 1989). When serine is substituted for Lys533, the amino acid side chain is shortened by two carbons, and the positive charge is omitted. Despite a 580-fold reduction in enzymatic activity, the apparent p*K* of the ^{31}P chemical shift of the K533S mutant enzyme is similar to that of wild-type enzyme; the total chemical shift change, however, is reduced from 2.88 to 1.32 ppm (Figure 2A, Table I). The similar apparent p*K* values for wild-type and K533S mutant enzyme suggest that a salt bridge or a hydrogen bond still links Ser533 and cofactor phosphate. Model building studies using the R-state of muscle phosphorylase predict that such a hydrogen bond can only be formed when the cofactor pyridine ring moves toward the serine residue by rotation of bond angles at the Lys645 side chain and the 5′-phosphate group (Figure 4). Slight changes in torsion angles around the phosphorus atom can be envisioned to be responsible for the observed reduction in the total ^{31}P chemical shift of pyridoxal-P in K533S mutant enzyme. The presence of arsenate alters the ^{31}P chemical shift only very slightly, indicating that optimal alignment of the cofactor phosphate group facilitated by Lys533 seems to be important for catalysis. Although 100 mM Li^+ can partially restore the enzymatic activity of the K533S mutant enzyme (Schinzel, 1991), addition of this cation does not alter the ^{31}P resonance. The partial reactivation of the mutant enzyme (9-fold) might be too small to be detected by the NMR method.

The Y538F mutant enzyme (Tyr573 in rabbit muscle phosphorylase) with 12% residual activity is the most active species of the five mutant enzymes tested. The pH dependence of the ^{31}P chemical shift of the cofactor phosphate of the Y538F mutant enzyme is very similar to that of wild-type enzyme (Figure 2A, Table I), suggesting that the hydroxyl

group of Tyr538 is not essential for catalysis and mainly involved in substrate binding. These conclusions are also corroborated from steady-state kinetics (Schinzel & Palm, 1990).

The interpretation of changes in the ^{31}P chemical shift of pyridoxal-P upon substitution of Arg534 or Glu637 (Arg569 or Glu672 in rabbit muscle phosphorylase) requires that interactions with other neighboring amino acid residues should be considered. However, a detailed interpretation cannot be given because original 3-D structural data for *E. coli* phosphorylase are not yet available. The 3-D structure of muscle phosphorylase in the R-state reveals hydrogen bond distances of Arg534 and Lys539 to sulfate which in turn is in hydrogen bond distance to the cofactor phosphate (Figure 1). The γ-carboxylate of Glu637, however, is at least 5 Å distant from the sulfate, cofactor phosphate, Arg534, and Tyr538. Only the ϵ-amino group of Lys539 is in the 4–5-Å distance range whereas Arg534 can form a hydrogen bond to Tyr538. In contrast, in the heptulose 2-phosphate complex of muscle phosphorylase, Glu672 is in hydrogen bond distance to Lys574 and the 2-OH and 3-OH groups of the sugar (Johnson et al., 1990). The ^{31}P NMR data of unliganded E637D mutant enzyme suggest that in this species the cofactor behaves similar to that of the free pyridoxal-P model Schiff base ($pK = 6.4$). This result then would indicate that some contacts of the cofactor phosphate to the active-site residues are disturbed, i.e., most probably to Lys533. Since Glu637 is not in hydrogen bond distance to the cofactor phosphate, changes introduced by mutating this residue have to be transmitted to the cofactor phosphate by other amino acid residues. The only residue close enough for a strong electrostatic interaction with Glu637 in the R-state is Lys539. Substitution of the Glu637 by an aspartate reduces the length of the side chain by about 1 Å but retains the charge. Hence, the electrostatic interaction of Asp637 with Lys539 may possibly pull the positive charges of Lys539, Arg534, and consequently Lys533 away from the cofactor phosphate, thereby breaking the hydrogen bond of Lys533 and distorting the bond angles around the phosphorus atom. This finding may be caused by other conformational rearrangements which could only be proven by X-ray structure of the E637D mutant enzyme. The apparent pK of the transition found for R534Q suggests that the hydrogen bond between Lys533 and the cofactor phosphate is intact. Therefore, changes in the O–P–O bond angles of the pyridoxal-P may be transmitted via the substrate phosphate cofactor phosphate contacts. The apparent pK of the ^{31}P chemical shift of pyridoxal-P of the E637Q mutant enzyme is significantly different from all other cases discussed. Therefore, the interpretation used for the other mutant enzymes cannot be employed for this species. However, the replacement of the negative charge at Glu637 by a neutral ligand accompanied by a remarkably different behavior of the unliganded E637Q mutant enzyme (Figure 2B) and the reversal of the cofactor phosphate transition in the presence of arsenate (Figure 3) point toward a role of the negative charge of Glu637 in the hydrogen bond and charge network of the active site.

The cofactor phosphate reveals in the presence of arsenate a pH dependence with apparent pK values of 6.7 and 6.8 for wild-type and E637Q mutant enzyme, respectively. The transition is reversed in one of these cases, indicating small but significant differences in the O–P–O bond angles. The chemical shift of the cofactor phosphate of the E637D mutant enzyme in the presence of 60 mM arsenate was found to be less affected by pH in the range from 6.1 to 8.5. In this binary complex, the interaction of cofactor phosphate and substrate anion is impeded. Hence, loss of enzymatic activity in these mutant enzymes appears to be due to impaired coupling of cofactor phosphate and substrate anion.

In conclusion, for all mutant phosphorylases with relevant loss of enzymatic activity, significant changes of the ^{31}P chemical shift of the cofactor phosphate were observed. With the exception of the Y538F mutant enzyme, the total chemical shift of all other mutant enzymes was significantly reduced, implying changes in torsion angles around the cofactor phosphorus. The unchanged apparent pK values of the cofactor phosphate transition following the substitution of one of the positively charged amino acid residues (K533S, R534Q), however, illustrate that the hydrogen bonds of the cofactor phosphate to active-site residues are not disrupted. In contrast, effects on the chemical shift of the cofactor phosphate upon removal (E637Q) or withdrawal (E637D) of the negative charge at the active site strongly suggest an indirect interaction between Glu637 and the cofactor phosphate. Therefore, despite its difficulties in interpretation, ^{31}P NMR spectroscopy of *E. coli* phosphorylases can provide information about subtle conformational changes around pyridoxal-P as a prerequisite for catalysis. We hope to give a more conclusive interpretation of ^{31}P NMR data of *E. coli* phosphorylase when more mutant enzymes have been studied and possibly 3-D structural data become available.

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Registry No. Lys, 56-87-1; Tyr, 60-18-4; Ser, 56-45-1; Glu, 56-86-0; Arg, 74-79-3; maltodextrin phosphorylase, 9035-74-9; pyridoxal-P, 54-47-7.

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Peptidylglutamyl–Peptide Hydrolase Activity of the Multicatalytic Proteinase Complex: Evidence for a New High-Affinity Site, Analysis of Cooperative Kinetics, and the Effect of Manganese Ions†

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ABSTRACT: The multicatalytic proteinase (MCP) complex or proteasome is a major nonlysosomal proteinase of eukaryotic cells. The proteinase can cleave peptide bonds on the carboxyl side of hydrophobic, basic, or acidic amino acid residues. These activities have been referred to as "chymotrypsin-like", "trypsin-like", and "peptidylglutamyl–peptide hydrolase" activities, respectively, and have been shown to be catalyzed at distinct sites. The latter activity is often assayed with the synthetic peptide substrate Z-Leu-Leu-Glu-β-naphthylamide (LLE-NA). *N*-tBoc-Ala-Ala-Asp-SBzl is also a substrate for the rat liver MCP, suggesting a broader specificity for cleavage on the carboxyl side of acidic residues than the peptidylglutamyl–peptide hydrolase activity previously reported. The pH optimum is in the range of pH 7.0–7.5. Studies of the dependence of velocity on LLE-NA concentration show (a) that there is a high-affinity site (LLE1) which obeys Michaelis–Menten kinetics with a K_m value of ~100 μM and (b) that at higher substrate concentrations (LLE2) the curve is sigmoidal, suggesting either allosteric activation of the proteinase at a second site or the involvement of multiple catalytic sites which display positive cooperativity. Activity at the high-affinity site (LLE1) can be distinguished from that of the activity of the LLE2 component by the effect of inhibitors, divalent metal ions, and KCl, as well as by its response to heat treatment. The addition of 1 mM $MnCl_2$ stimulates both LLE1 and LLE2 activities and also permits saturation of MCP with substrate at concentrations of LLE-NA below the solubility limit of this peptide. Under these conditions, the Hill coefficient calculated for LLE2 is 5.1 and the $K_{0.5}$ value is 0.28 mM. Such activation of the MCP complex at high substrate concentrations should be advantageous for the regulation of proteinase activity within cells and for the rapid degradation of protein substrates.

The multicatalytic proteinase (MCP),[1] prosome, or proteasome is a complex multisubunit proteinase which is found in the nucleus and cytoplasm of eukaryotic cells [see Rivett (1989a) and Orlowski (1990) for reviews]. It appears to be involved in ubiquitin-dependent as well as ubiquitin-independent nonlysosomal pathways of protein degradation (Eytan et al., 1989; Driscoll & Goldberg, 1990; Rivett, 1990; Heinemeyer et al., 1991).

The proteinase has a hollow cylindrical structure, composed of at least 24 subunits. The number of different types of polypeptide, each with a molecular mass in the range 22–34 kDa, depends on the species. The simplest proteinase, composed of only two different types of subunits, has been isolated

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[1] Abbreviations: tBoc, *tert*-butyloxycarbonyl; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LLE1, high-affinity site assayed at 0.1 mM LLE-NA; LLE2, lower affinity site(s) assayed at 0.4 mM LLE-NA; LLE-NA, Z-Leu-Leu-Glu-β-naphthylamide; MCP, multicatalytic proteinase; SBzl, thiobenzyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Z, benzyloxycarbonyl.

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