Escherichia coli Maltodextrin Phosphorylase: Contribution of Active Site Residues Glutamate-637 and Tyrosine-538 to the Phosphorolytic Cleavage of α-Glucans†

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ABSTRACT: The role of *Escherichia coli* maltodextrin phosphorylase (EC 2.4.1.1) active site residues Glu637 and Tyr538 which line the sugar–phosphate contact region of the enzyme was investigated by site-directed mutagenesis. Substitution of Glu637 by an Asp or Gln residue reduced k_{cat} to ≈0.2% of wild-type activity, while the K_m values were affected to a minor extent. This indicated participation of Glu637 in transition-state binding rather than in ground-state binding. ^{31}P NMR analysis of the ionization state of enzyme-bound pyridoxal phosphate suggested that Glu637 is also involved in modulation of the protonation state of the coenzyme phosphate observed during catalysis. Despite loss of proposed hydrogen-bonded substrate contacts, the Tyr538Phe mutant enzyme retained more than 10% activity; the apparent affinity of all substrates was slightly decreased. Mutations at either site affected the error rate of the enzyme (ratio of hydrolysis/phosphorolysis). Besides a role in substrate binding, the hydrogen-bond network of Tyr538 supports the exclusion of water from the active site.

Pyridoxal 5′-phosphate (PL-*P*)[1] dependent α-glucan phosphorylases (EC 2.4.1.1) catalyze the first step in the mobilization of storage polysaccharides, resulting in the energy-conserving release of glucose 1-phosphate (Glc-1-*P*):

$$(\alpha\text{-1,4-glucoside})_n + P_i \rightleftharpoons (\alpha\text{-1,4-glucoside})_{n-1} + \alpha\text{-D-Glc-1-}P$$

The advantage nature gains in the phosphorylase reaction by making use of the phosphorolytic principle in the breakdown of a macromolecule instead of hydrolytic cleavage is clearly reflected by the key role phosphorylases play as the ultimate target enzyme of metabolic, hormonal, or neural controls of glycogen metabolism.

Beyond the aspects of control mechanisms, the rather unique features of the phosphorolytic degradation of macromolecules make phosphorylases an attractive model to ask if the mechanism can give an answer to the question how a biological catalyst copes with the problem of a nonhydrolytic cleavage and energy conservation in an aqueous environment.

Kinetic studies with pyridoxal 5′-*P* analogues and a new class of "glycosylic" substrate analogues as well as structural studies by ^{31}P NMR and X-ray crystallography provided a clue to the chemical mechanism of phosphorolysis [for recent reviews, see Madsen and Withers (1986), Johnson et al. (1989), and Palm et al. (1990)]. Two alternate models for the phosphorylase mechanism have been proposed, which both require highly coordinated phosphate residues of the coenzyme and the substrate. One mechanism relies on a constrained phosphate residue of PL-*P* creating an electrophilic trigger for the attack to Glc-1-*P* (Withers et al., 1982; Madsen & Withers, 1986), while the other mechanism relies on the cofactor phosphate as an acid–base catalyst promoting the protonation of the substrate phosphate (Klein et al., 1984, 1986; Palm et al., 1990). The latter mechanism shares a number of chemical and stereochemical similarities with preferentially "hydrolytic" glucosyltransferases (Hehre et al., 1986; Chiba et al., 1988), but it differs in its requirement for PL-*P*, its dependence on a "mobile" phosphate anion, and its apparent reversibility.

The structural basis for understanding the catalytic mechanism was provided from in situ X-ray studies of product formation from heptenitol and orthophosphate in the crystal (McLaughlin et al., 1984; Hajdu et al., 1987). The structure of the phosphorylase *b*–heptulose 2-*P* complex was recently refined and displays an arrangement of interacting groups when the reaction proceeds in the direction of "glycogen degradation" (Johnson et al., 1990). The transition-state intermediate-like performance of the heptulose-2-*P* complex provides a special advantage for molecular interpretation of the catalytic mechanism.

While previous biochemical studies aimed primarily at elucidating the nature of the catalytic mechanism in terms of understanding the basic chemical principles, the structure–function relations, which let phosphorylases favor phosphorolysis over hydrolysis, remain to be established. On the basis of the 3-D structure, a critical role of glutamic acid and tyrosine residues lining the catalytic site proximal to the glucopyranosyl ring and the "substrate" phosphate (Figure 1) was anticipated. Since their functional contribution is still under discussion, this paper aims at establishing the principles of the structure–function relations of these amino acid side chains.

The high sequence homology (92–100%) of domains involved in substrate binding and catalysis among the glycogen phosphorylases (Palm et al., 1985, 1986; Newgard et al., 1989) allowed us to probe the function of the corresponding Glu637 and Tyr538 in maltodextrin phosphorylase by site-directed mutagenesis. The *Escherichia coli* maltodextrin phosphorylase is especially well suited for these experiments since, unlike mammalian phosphorylases, it is an unregulated enzyme.

Materials and Methods

Strains and Plasmids. *E. coli* strain pop2158 [Δ*mal*A518, F⁻, *ara*D139, Δ*lac*U169, *rps*L, *rel*A, *thi*A] was obtained from Dr. M. Schwartz, Institut Pasteur, Paris (Raibaud et al.,

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[1] Abbreviations: Glc-1-*P*, α-D-glucose 1-phosphate; PL-*P*, pyridoxal 5′-phosphate.

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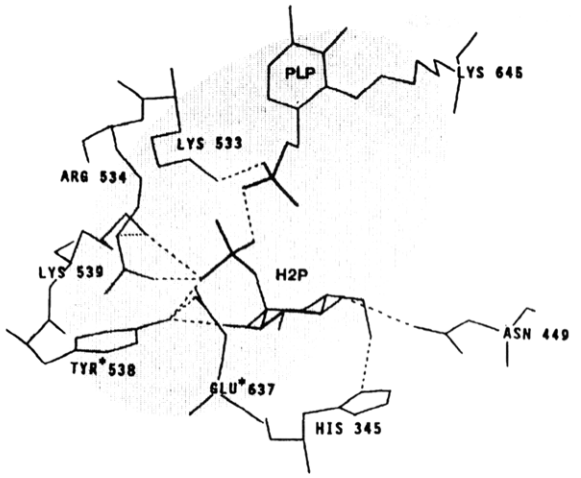

	1	3	4	55	55	6	6*P*
	3	7	8	66	77	7	8*L*
	5	7	4	89	34	2	0*P*
RabP	-LGNGGLGRL-	-TNHTVLPEA-	-TNGITPRRW-	-QVKRIHEYK-		-EASGTGNMK-	
EcoM	-LGNGGLGRL-	-TNHTVLPEA-	-TNGITPRRW-	-QIKRLHEYK-		-EASGTGNMK-	
	1	3	4	55	55	6	6*P*
	1	4	4	33	33	3	4*L*
	4	5	9	34	89	7	5*P*

FIGURE 1: Partial view of amino acid residues forming the active site in a prototype phosphorylase. The model is based on the structure of the rabbit muscle phosphorylase *b*–heptulose 2-*P* complex (Hajdu et al., 1987) but uses the numbering of *E. coli* maltodextrin phosphorylase. The alignment of relevant amino acid residues is given in the bottom section. Hydrogen bonds are shown by dotted lines. (*) Mutagenized amino acid residues, described in this study. H2P, heptulose 2-phosphate.

1983). Plasmid pMAP101 was constructed from plasmid pOM13 as described before (Bloch & Raibaud, 1986; Palm et al., 1986). The following strains used for mutagenesis were kindly provided by Dr. H. J. Fritz, Max-Planck-Institut für Biochemie, Martinsried, FRG: *E. coli* BMH71-18:[Δ(*lac-pro*AB), *thi*, *sup*E; F′, *lac*I^q, *lac*ZΔM15, *pro*A$^+$B$^+$], BMH71-18mutS:[BMH71-18 *mut*S215:Tn*10*], MK30-3:[Δ(*lac-pro*AB), *rec*A, *gal*E, *str*A; F′, *lac*I^q, ZΔM15, *pro*A$^+$B$^+$], and phage M13mp9rev.

DNA Procedures. DNA was manipulated by standard procedures (Maniatis et al., 1982). Restriction endonucleases, T4 DNA ligase, and polynucleotide kinase were used as recommended by the manufacturer (Boehringer Mannheim). Transformation of *E. coli* was performed by the method of Hanahan (1985). Oligonucleotides were prepared on an Applied Biosystem 380A DNA synthesizer and purified by preparative electrophoresis on a denaturing 20% polyacrylamide gel.

Mutagenesis. (*A*) *Tyr538Phe.* A 600-bp *Bgl*II–*Pst*I fragment from the *mal*P gene was subcloned into the *Bam*HI and *Pst*I site of M13mp9. Site-directed mutagenesis was accomplished by the procedure of Kramer and Fritz (1987) with a 23-bp oligonucleotide (GTTTGCACGAGTTTAAACGCCAG) in which the TAC codon for Tyr538 was replaced by a TTT codon for Phe. This exchange, in addition, created a *Dra*I recognition site to facilitate screening. To ensure that no secondary priming site existed, the same oligonucleotide was used as a sequencing primer. Only one priming site was found. A *Bst*XI–*Pst*I fragment containing the mutation was recloned into *Bst*XI–*Pst*I-cleaved plasmid pMAP101. Transformants were selected by iodine staining for glycogen accumulation (Schwartz, 1967).

(*B*) *Glu637Asp.* The unique *Hin*dIII site of the *mal*P gene in plasmid pMAP101 was cleaved, and protruding ends were removed by digestion with mung bean nuclease (Pharmacia, Uppsala, Sweden), and a *Sal*I linker was ligated into the newly created blunt-end site (Figure 2). After *Sal*I cleavage, the

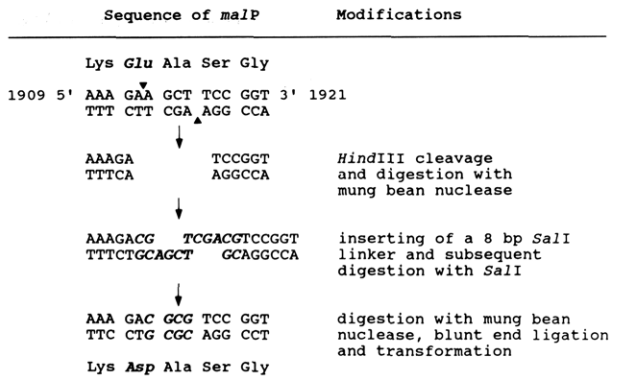

FIGURE 2: Mutagenesis of Glu637 in maltodextrin phosphorylase. The insertion of an Asp residue for Glu637 by modification of the *Hin*dIII restriction site at position 1913 of the *E. coli mal*P gene is shown [adapted from Palm et al. (1987)]. Inserted base pairs are given in italics.

protruding ends were digested as before, and the plasmid was religated and transformed into *E. coli* pop2158. This procedure converted Glu to an Asp residue and removed the *Hin*dIII site, while creating a new *Mlu*I site.

(*C*) *Glu637Gln.* The mutation was introduced by replacing the DNA fragment between the *Pst*I and *Asp*718 site of pMAP101 with a synthetic DNA linker. This linker allowed the original GAA (Glu) codon to be changed to a CAG (Gln) codon and introduction of a unique *Stu*I site by a silent GCT to GCC exchange. The two complementary oligonucleotides (GGTAAACAGGCCTCCG and GTACCGGAGGCCTGTTTACCTGCA) were hybridized by incubation in 10 mM Tris–acetate, pH 7.4, 50 mM KCl, and 2 mM EDTA for 3 min at 100 °C and then slowly cooled to room temperature. Ten picomoles of this linker was ligated into 2 pmol of *Asp*718/*Pst*I-cleaved pMAP101 and transformed into *E. coli* pop2158. The nucleotide exchanges were confirmed by sequencing (Sanger et al., 1977).

Enzyme Purification. E. coli pop2158 carrying plasmid pMAP101 or mutagenized derivatives were grown in LB medium supplemented with 0.4% maltose for 24 h. Washed cells were disrupted by sonication or by repeated passage through a French press. The cell-free extracts of mutant enzymes were further purified as described for the wild-type enzyme (Schächtele et al., 1978). Purification of low-activity mutant proteins was followed by SDS–PAGE. PL-*P* was determined as described by Wada and Snell (1971). Protein was measured by the method of Bradford (1971) or from the absorbance at 280 nm, with $E^{1cm}_{0.1\%} = 1.36$. UV spectra were recorded on a Beckmann DU64 spectrometer.

Kinetic Measurements. Enzyme activity was assayed at 30 °C in the direction of phosphorolysis in a coupled assay (Helmreich & Cori, 1964) in 50 mM Tris–acetate buffer, pH 6.9, containing 10 mM maltoheptaose (generously provided by Boehringer Mannheim) or 2% dextrin, 10 mM KH_2PO_4, 1 mM NAD^+, 5 mM $MgCl_2$, 1 mM EDTA, 5 μM glucose 1,6-bis-*P*, 2 μg/mL phosphoglucomutase, and 2 μg/mL *Leuconostoc mesenteroides* glucose-6-*P* dehydrogenase. Arsenolysis and hydrolysis were measured by a spectrophotometric glucose assay in 25 mM MES buffer, pH 6.9, with 40 μg/mL glucose dehydrogenase (Merck, Darmstadt, FRG) and 1 mM NAD^+. In the direction of synthesis, the enzyme was assayed in 25 mM MES buffer, pH 6.9, 10 mM Glc-1-*P*, and 5 mM maltotetraose. The release of P_i was measured by the method of Saheki et al. (1985). Initial velocity studies were performed at 30 °C by varying the concentration of the substrates within a 10-fold range.

The kinetic parameters were determined by nonlinear regression with the ENZFITTER program (Leatherbarrow, 1987). Changes in free energy were calculated with $\Delta G^{\circ\prime} = -RT \ln (k_{cat\ mut}/K_{m\ mut})/k_{cat\ wt}/K_{m\ wt})$ (Fersht et al., 1985).

NMR. Fourier transform ^{31}P NMR spectra were performed at 72.86 MHz on a Bruker WH-180 wide-bore superconducting spectrometer as described by Schnackerz et al. (1979). Enzyme samples (2.1×10^{-4} M in 10 mL of 50 mM MES, 50 mM KCl, 1 mM DTT, and 1 mM EDTA) in 20-mm tubes, enclosing a 5-mm concentric tube with D_2O, were recorded at 26 °C for 12 h.

RESULTS

Construction of Mutants. The gapped-duplex approach of site-directed mutagenesis (Kramer & Fritz, 1987) was used to replace Tyr538 by a Phe. Approximately 10–20% of the plaques grown on the *sup*E$^-$ background were identified as the desired nucleotide exchange. Glu637 was converted to Asp by inserting and modifying a *Sal*I linker into the opened and blunt-ended *Hin*dIII site at position 1913. All recombinants had lost the original *Hin*dIII site and contained a newly created *Mlu*I site. Introduction of a Gln residue for Glu637 was accomplished by replacing the 20-bp DNA fragment between the *Pst*I site, position 1905, and the *Asp*718 site, position 1925, with a synthetic fragment containing the Glu → Gln mutation.

Isolation and Purification of Mutant Phosphorylases. Wild-type and mutant phosphorylases were expressed under control of their own promotor in a *mal*P$^-$ strain (*E. coli* pop2158). The overexpressed wild-type or mutant enzymes accounted for 5–7% of the total soluble protein. Wild-type or mutant enzymes were purified from crude bacterial extracts in four steps by (I) Polymin P (BASF, Ludwigshafen) fractionation, (II) ammonium sulfate fractionation, (III) DEAE-Sephacel chromatography, and (IV) affinity chromatography on Sepharose-bound glycogen. Both mutant and wild-type enzymes exhibited a single band and identical mobilities under denaturating gel electrophoresis conditions.

The low activity of the Glu637Asp mutant could be caused by a contamination of an inactive enzyme with traces of wild-type phosphorylase. However, the observed differences in several catalytic properties, i.e., the relation arsenolysis/phosphorolysis and the rate of hydrolysis (see below) of mutant and parent enzyme, imply a correlation of the reported values with those of the mutant protein. Furthermore, both mutant enzymes lack the capability of the wild-type enzyme to turn over 2′-deoxyglucosyl (α-1,4-glucoside)$_n$, yielding glucal; this property was used to test for the absence of wild-type enzyme (Palm, Becker, and Schinzel, unpublished results). Another source of contamination could potentially come from the presence of a second α-glucan phosphorylase in *E. coli*. Maltodextrin phosphorylase and all mutant proteins eluted from DEAE-Sephacel at an ionic strength of about 0.1 M NaCl, which is significantly lower than the value of 0.35 M NaCl reported for α-glucan phosphorylase (Chen & Segal, 1968). Substrate specificity and kinetic parameters of the *glg*P gene product as deduced from the overexpressed gene (Yu et al., 1988; Romeo et al., 1988; Choi et al., 1989) differed widely from those of maltodextrin phosphorylase. Both activities can be separated by DEAE ion-exchange chromatography in the course of the standard purification procedure.

An affinity for maltodextrin substrates was retained by all mutants as shown by retention of the mutant enzymes on glycogen affinity columns and their retarded migration on native polyacrylamide gels in the presence of 1% dextrin (not shown). The UV spectra of mutant phosphorylases were identical with the spectrum of the wild-type enzyme. Specifically, the $OD_{330/280}$ ratio (1:26) was not changed in mutant phosphorylases, when compared with that of the wild type, indicating that PL-*P* binding was not altered by the mutations. Determinations of the coenzyme content yielded values of 0.97–0.99 mol of PL-*P* per subunit of 90 500 Da. Most remarkably, both the overexpressed wild-type and mutant phosphorylases contained the full equivalent of covalently bound PL-*P*. The p*I* of both mutant and wild-type enzymes was determined on agarose gels (pH gradient 3–7) to be 6.5. The mutant phosphorylases were equally reactive with antibodies raised against wild-type enzyme, yielding identical antiserum titers in an ELISA (1:6400). From all these experiments there was no evidence for any major structural changes. However, the phosphorylase is quite a large enzyme, and we are aware that minor changes might not be assessed by the methods used.

Table I: Kinetic Properties of Mutant and Wild-Type Phosphorylases[a]

	kinetic parameters				
	phosphorolysis			synthesis (Glc-1-*P*)	
	K_m (mM)				
enzymes	P_i	G_7	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
wild type	0.50	0.50	28	1.0	39
Tyr538Phe	0.87	0.73	3.3	2.8	12.5
Glu637Asp	0.55	0.40	0.05	2.3	0.06
Glu637Gln	0.52	0.44	0.03	0.6	0.04

[a] Kinetic parameters of wild-type and mutant phosphorylases were determined under comparable conditions except that the enzyme concentrations were adjusted. The error range was smaller than 5% for wild-type enzyme and smaller than 15% for mutant enzymes. G_7, maltoheptaose; P_i, orthophosphate.

Kinetic Properties. A rapid equilibrium random bi bi mechanism has been proposed for phosphorylase (Engers et al., 1970). This scheme predicts linear steady-state kinetics if one substrate is varied at fixed concentrations of the second substrate, which is consistent with the present study. Apparent K_m and k_{cat} values at fixed concentrations of the second substrate, approaching saturation, are listed in Table I. The Glu637Asp mutation reduced the k_{cat} values by a factor of 600 in both directions. K_m values for orthophosphate and oligosaccharides remained unchanged, whereas the K_m value for Glc-1-*P* was increased about 2-fold.

The activity of the Glu637Gln mutant is further reduced in both directions by a factor of 2 when compared with that of the Glu637Asp exchange and by a factor of 900 when compared with that of the wild-type enzyme, while the K_m values are in the same range as for the wild-type and Glu637Asp enzymes.

In comparison, the mutation Tyr538Phe had a small effect on k_{cat}, lowering it by a factor of 9; K_m values increased slightly for the substrates P_i, Glc-1-*P*, and maltoheptaose.

To test the effect of the mutations on the pH profile of the reaction, we looked for differences in the pH dependence of k_{cat} and K_m of wild-type and mutant enzymes. The relative activity and the relative K_m of all three mutants remained unchanged in the pH range from 6.2 to 8.5, with a pH optimum between 6.5 and 6.9. In the direction of phosphorolysis no reliable data below pH 6.2 could be obtained, since the rate of the coupled assay became rate limiting. In the synthesis direction, too, the pH dependence of the Glu637Asp mutant was comparable to that of the wild-type enzyme between pH 5 and pH 8.

Arsenate can be treated kinetically as a slowly reacting substrate analogue or as a competitive inhibitor of orthophosphate. In the first case, we assayed arsenolysis of mal-

Table II: Kinetic Properties of Wild-Type and Mutant Phosphorylases with Arsenate as Substrate[a]

enzyme	$k_{cat\ As}$ (arsenolysis) (s^{-1})[b]	$k_{cat\ As}/k_{cat\ P_i}$	K_i (arsenate) (mM)[c]
wild type	3.30	1:8.5	0.54
Tyr538Phe	0.43	1:7.5	0.54
Glu637Asp	≤0.001[d]	1:50[d]	0.84

[a] The error range was smaller than 15% for all values. [b] Arsenolysis was measured as the release of glucose from maltoheptaose in the presence of arsenate. [c] The K_i of arsenate was determined by measuring the K_m values for P_i (0.5–15 mM) in the presence of 0, 1, and 2.5 mM arsenate. [d] This value represents an upper limit due to the simultaneous recording of glucose released by hydrolysis.

Table III: Error Frequency of Wild-Type Phosphorylase Compared to That of Mutant Phosphorylases[a]

enzyme	k_{cat} (s^{-1}) phosphorolysis	hydrolysis[b]	error frequency
wild type	28	0.0032	1:9000
Tyr538Phe	3.3	0.0062	1:530
Glu637Asp	0.05	0.001[c]	1:50[c]

[a] The error range was smaller than 15% for phosphorolysis and smaller than 20% for hydrolysis. [b] Release of glucose from maltoheptaose was determined in the absence of inorganic phosphate by glucose dehydrogenase and NAD. [c] Values differ from a factor of 2 from batch to batch.

todextrins by the glucose dehydrogenase linked spectrophotometric determination of glucose, which is derived from the spontaneous hydrolysis of glucose 1-arsenate. Relative affinities for arsenate were determined from K_m or, in the presence of P_i, from K_i. The ratio arsenolysis/phosphorolysis is 1:8 for both wild-type phosphorylase and the Tyr538Phe enzyme. However, for the Glu637Asp mutant, the arsenolysis rate dropped by a factor of >50 (Table II). Recording of the low arsenolytic activity was restricted due to interference by glucose resulting from hydrolysis (see below). K_i values for competitive inhibition of phosphorolysis by arsenate are comparable in size to the K_m, with a small but significant increase for the Glu637Asp mutant.

Hydrolytic Activity of Phosphorylase. As a measure of hydrolytic activities, we determined the release of glucose from maltoheptaose in the absence of P_i (Table III). Within the limits of the analytical methods, the apparent hydrolysis rate was independent of the presence or absence of P_i. To exclude any contamination by glucosidases or amylases, the reaction products were also analyzed by thin-layer chromatography for the absence of maltooligosaccharides smaller than four glucose residues, commonly arising from the hydrolytic action of the former enzymes. Apparent hydrolysis was totally inhibited by gluconolactone, which is an inhibitor of glucosidases and phosphorylases but was not inhibited by the specific glucosidase and amylase inhibitor acarbose (Gold et al., 1971; Truscheit et al., 1981).

The ratio of rates of glucose vs Glc-1-*P* formation was very low in the wild-type phosphorylase and increased 17-fold in the Tyr538Phe mutant enzyme and 180-fold in the Glu637Asp mutant. This means that the hydrolytic activity of the Tyr538Phe mutant even doubled in absolute terms in comparison to that of the wild-type enzyme. Increased hydrolytic activity of the mutant enzymes was also observed with [U-^{14}C]Glc-1-*P* as substrate and with analysis of the products by TLC and autoradiography. Controls were made for the action of phosphatases.

NMR. ^{31}P NMR spectra were recorded at pH 7.2 for both wild-type and the Glu637Asp mutant enzyme in the absence of substrates. The Glu637Asp mutant enzyme showed a single resonance with a shift of 2.4 ppm compared to 3.6 ppm for the wild-type phosphorylase (Figure 3). This downfield shift indicates that the protonation equilibrium in the mutant enzyme is shifted to a more protonated form.

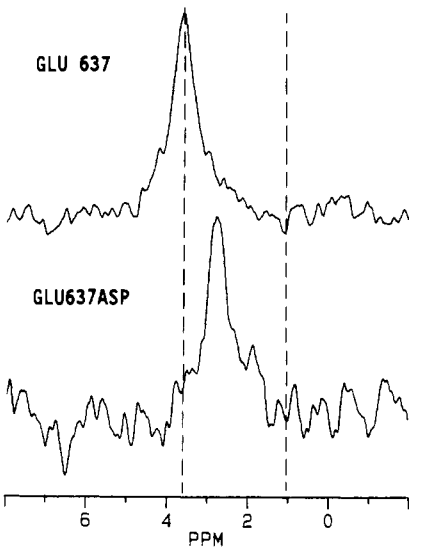

FIGURE 3: ^{31}P NMR spectra of PL-*P* in wild-type and the Glu637Asp mutant maltodextrin phosphorylase at pH 7.2 in the absence of ligands. Broken lines indicate spectral shifts characteristic for the dianionic and monoanionic forms in wild-type phosphorylase.

Discussion

The structure of the phosphorylase *b*–heptulose 2-*P* complex makes an important contribution toward a molecular comprehension of interacting sites in the interpretation of the phosphorylase mechanism. The most attractive feature of this enzyme–substrate complex is the close phosphate–phosphate coordination, contributed by the cofactor and substrate phosphates and which bears the answer to the enigma of the role of PL-*P* in phosphorylases. The interaction of two phosphates was first noted by Parrish et al. (1977) and structurally confirmed by McLaughlin et al. (1984). The mechanism of this interaction was assigned from kinetics and ^{31}P NMR with PL-*P* and glycosylic substrate analogues (Klein et al., 1984, 1986). On the basis of this structure five prominent amino acids are anticipated which could render possible the unique "catalytic" arrangement of cofactor and substrates. Three of them (Lys568, Arg569, and Lys574) provide a cluster of positively charged amino acid side chains at the proximal site of the catalytic center in hydrogen-bond distance to the cofactor and substrate phosphates. An essential role of these residues was verified by site-directed mutagenesis of the corresponding amino acids in maltodextrin phosphorylase (Palm et al., 1990; Schinzel et al., unpublished results). Two more charged or polar residues, Glu672 and Tyr573, are outstanding for their ability to form hydrogen bonds to the glucose residue and might be involved in binding and catalysis.

In a first approach to study a participation of Glu672 in catalysis, the Glu637 residue of maltodextrin phosphorylase was substituted by an Asp, which shortens the side chain by about 1 Å while retaining the charge. In a second approach Glu was changed to the isosteric but neutral Gln. Model building predicts that the distances allowing for hydrogen bonds to the glucosyl residue should be less affected by the Glu637Asp exchange than would be inferred from the withdrawal of a methyl group without further adjustments. Both mutations caused a great reduction of k_{cat}. In contrast, there were no (for P_i and maltoheptaose) or small (for Glc-1-*P*)

changes in the apparent affinities (Table I).

One possible explanation for the loss of activity in these mutant enzymes would be to consider Glu637 a direct proton donor for catalysis. However, in the crystal structure (Figure 1), the γ-carboxyl group of Glu637 seems too far from either the O5 or C1 atom of the sugar moiety for direct interactions. In keeping with this argument, substitution of Glu637 by a neutral or acidic residue indiscriminately yielded approximately the same decrease in k_{cat}. This was not expected for substitution by an aspartic acid side chain which, in principle, could directly or via a water molecule step in as proton donor. The latter case was observed for the established proton donor of T4 lysozyme (Anand et al., 1988), where a relatively high level of activity was retained if Glu11 was replaced by an Asp and where no measurable activity occurred after exchange by Gln. If an essential proton donor is removed by site-directed mutagenesis, one might expect a partial increase of activity at pH values lower than the pH optimum of the wild type, as was observed for dihydrofolate reductase (Howell et al., 1986). This was not found for the Glu637Asp mutant enzyme.

Alternatively, the Glu637 carboxylate anion could promote or stabilize the proposed intermediate oxocarbonium ion at the C1 atom (Madsen & Withers, 1986; Palm et al., 1990) by ionic interactions as described for lysozyme or by formation of a covalent intermediate as suggested for β-galactosidase (Cupples et al., 1990). However, on the basis of its steric disposition at the si side of the glucopyranose ring, Glu637 is in an unfavorable position to allow for an S_N2 double-displacement mechanism to establish retention of α-configuration. Regarding the extent of effects on ionic stabilization of oxocarbonium ions by mutations, significant residual activities were observed when a carboxylate anion was replaced with a neutral amino acid in the case of lysozyme (Malcolm et al., 1989; Anand et al., 1988). Reductions of rates were considerably larger in the case of the Glu637Asp and Glu637Gln mutants. Even larger reductions were observed for the exchanges of the established nucleophile Glu461 in β-galactosidase, which is proposed to make a covalent intermediate (Cupples et al., 1990). While the steric requirements in phosphorylase make a covalent intermediate unlikely, the involvement of Glu637 in ionic stabilization is also judged improbable by the rate effect following mutagenesis. Since there is no other anion in the vicinity, the inorganic phosphate remains a candidate to stabilize the proposed intermediate oxocarbonium ion at C1 (Palm et al., 1990).

Disengagement of a hydrogen bond between Glu637 and a hydroxyl group of Glc-1-*P* might lead to loss of binding energies. Thus, the 3′-deoxy analogue of glucose and Glc-1-*P* was found to be less stably bound to phosphorylase *b* by 3 kcal/mol in the ground state (Street et al., 1986) and 6 kcal/mol in the transition state (Street et al., 1989). Since the change in the K_m value for Glc-1-*P* is relatively small for the Glu637Asp substitution, this mutation seems not to influence binding in the ground state. The reduction of k_{cat} makes it more likely that the mutation predominantly affects stabilization of the transition-state intermediate. The predicted structure of the transition state is based on the X-ray structure of the phosphorylase *b*–heptulose 2-*P* complex: Different to that in the single-crystal structure of Glc-1-*P*, the C1–O1–P bond is rotated in the potential transition-state analogue heptulose 2-*P*. This directs the substrate phosphate toward the 5′-phosphate of PL-*P*, providing the phosphate–phosphate contacts necessary for catalysis (Johnson et al., 1990). To force Glc-1-*P* into an equally reactive conformation, the enzyme has to provide a rigid glycosyl binding site. Mutations in this site, as in the present case, should affect the torsion of the C1–O1–P bond. This can explain the rate effects.

Long-range interactions of the Glu637 residue beyond next-neighbor contacts are revealed by ^{31}P NMR analysis. Phosphorus NMR has the potential to resolve the ionization state of PL-*P* and to signal the reversible dianion/monoanion interconversion in response to substrate binding and catalysis. We found that in the unliganded mutant enzyme the phosphate of PL-*P* is in a more protonated state than in wild-type phosphorylase (Figure 3). Since the dianionic form of PL-*P* is considered essential for activity of phosphorylases (Feldmann & Hull, 1977), this difference provided a very elucidating interpretation of how the mutation affects catalysis. On the basis of the structural constellation (Figure 1), it might be assumed, that the charge of Glu637 partially neutralizes the charge of Lys539 and the latter attenuates the protonation of PL-*P*. A replacement of Glu637 interferes with the balanced protonation of PL-*P* and the capability of Lys539 to modulate these contacts. The corresponding Lys574 in phosphorylase *b* is in hydrogen-bond distance to the Glu672 residue. In the binary complex, Lys539 together with Arg534 makes direct contacts to the substrate phosphate and stabilizes the phosphate–phosphate contact by establishing a network of positive charges. Replacement of the corresponding Lys539 in the *mal*P gene of *E. coli* creates a mutated protein, which either is not expressed or is rapidly degraded (Palm & Schinzel, 1989).

Complementary to these deductions, it appears not unexpected that the Glu637Asp mutant phosphorylase displays lower affinity for arsenate replacing the substrate phosphate.

In the phosphorylase *b*–heptulose 2-*P* complex, Tyr573, like Glu672, is in hydrogen-bond contact to at least one hydroxyl (2′-OH) of a glucosyl residue (Figure 1) and in addition makes direct contacts to the substrate phosphate. If hydrogen bonds of the tyrosyl OH group are relevant for binding of the binary complex in the ground as well as in the transition state, we might expect correspondingly large changes of K_m or k_{cat} when the hydrogen-bonding capability in maltodextrin phosphorylase is lost on conversion of Tyr to Phe. In the present case we observed only a small decrease in the apparent affinity for P_i and the oligosaccharide and a 2-fold increase of the K_m for Glc-1-*P*. The reduction of k_{cat} was small compared to that observed for the Glu–Asp exchange and represents a loss in apparent binding energy of about 1 kcal/mol. These values correspond fairly well to those expected from the loss of an uncharged hydrogen bond (Fersht et al., 1985). Therefore, Tyr538 seems to be mainly involved in binding of the glucopyranose ring, although contacts to the substrate phosphate are possible (Figure 1). Tyr573 was also considered to be a candidate for proton transfer in catalysis (Madsen & Withers, 1986). If this were true, a change to Phe should, therefore, affect the kinetic behavior much more strongly than was observed.

However, another consequence follows from replacement of Tyr by Phe in this very specific constellation. According to model building, the empty –OH site in the Tyr538Phe mutant would allow for "crystal water" next to the glucose residue and thus enhance "hydrolytic competition" to the essentially phosphorolytic reaction. Indeed, we found an increase in "error frequency" (see Table III). An even higher error ratio was observed for the Glu637Asp mutant (<100), but due to the much lower overall activity of this mutant, we were not able to measure these rates accurately. The observation that both rates can be inhibited simultaneously by phosphate analogues corroborates that hydrolysis is linked to phospho-

rylase activities. Both mutants behave more like sucrose phosphorylase, exhibiting high endogenous error rates (Silverstein et al., 1967). We conclude that only in the mutated enzymes is water no longer excluded from the catalytic site, allowing hydrolysis, which is independent of the presence of phosphate. These conclusions agree with the performance of native potato phosphorylase where isomerization without hydrolysis of the phosphate oxygens of Glc-1-*P* provides strong evidence against the presence of water near the active site (Kokesh & Kakuda, 1977).

As a result of our study, the function of some amino acid residues lining the sugar–phosphate contact region of maltodextrin phosphorylase became apparent. The Glu637 residue may function in a dual role: (i) Glu637 participates in transition-state binding, most likely through the 3′-OH group of the glucosyl pyranose, therefore providing the handle to lock Glc-1-*P* in a more favorable position for proton transfer. (ii) Additionally, Glu637 may be involved in the charge network promoting the active PL-*P* dianion form of phosphorylase. Quite unexpectedly, the hydroxyl group of Tyr538 is permissive as far as concerns glucosyl binding and catalysis. A much more important and specific function of this Tyr in phosphorylases may be to create a hydrophilic subsite with exclusion of water. Our results imply that the motif of a rigid, nonaqueous binding site cannot be changed without questioning the phosphorolytic reaction. This may explain why the catalytic site of phosphorylases is highly conserved throughout evolution (Newgard et al., 1989).

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Registry No. Glu, 56-86-0; Tyr, 60-18-4; maltodextrin phosphorylase, 9035-74-9; maltoheptaose, 34620-78-5; orthophosphate, 14265-44-2; α-D-glucose 1-phosphate, 59-56-3; arsenate, 15584-04-0.

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Asymmetric Short-Chain Phosphatidylcholines: Defining Chain Binding Constraints in Phospholipases†

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ABSTRACT: Several short-chain asymmetric lecithins with a total of 14 carbons in the acyl chains (ranging from 1-lauroyl-2-acetylphosphatidylcholine to 1-hexanoyl-2-octanoylphosphatidylcholine) have been synthesized and characterized. The specific activities of phospholipase A_2 from cobra venom, phospholipase A_2 from porcine pancreas, and phospholipase C from *Bacillus cereus* toward these lecithins as micelles have been determined. The results of these kinetic studies allow the definition of hydrophobic binding requirements in the active sites of these water-soluble phospholipases. For phospholipase C, with the exception of monomyristoylphosphatidylcholine, each of the asymmetric short-chain lecithins exhibits high activity, comparable to the 14-carbon symmetric short-chain species, diheptanoylphosphatidylcholine. Therefore, for phospholipase C, in addition to the acyl linkages, only a certain degree of hydrophobicity in the fatty acyl chains is requisite for substrate binding and appreciable hydrolysis; there is no chain specificity. The activity of phospholipase A_2 from cobra venom toward the same asymmetric lecithins is quite different. As the *sn*-2 chain lengthens, activity is increased to a maximum for diheptanoyl-PC. Further increase in the number of carbons in the *sn*-2 chain has no effect on hydrolysis rates. For this enzyme, seven carbons in the *sn*-2 chain are necessary for optimal activity. In contrast, porcine pancreatic phospholipase A_2 activity shows very little dependence on *sn*-2 chain length.

Symmetric short-chain lecithins have been used extensively as substrates for water-soluble phospholipases (DeHaas et al., 1971; Bonsen et al., 1972; Wells, 1974; Verger & De Haas, 1976; Little, 1977). These molecules can be monomeric in aqueous solution and hence are soluble substrates for lipolytic enzymes. As their concentration is increased, they form micelles rather than bilayer structures (Tausk et al., 1974a,b). Short-chain phospholipids are a good choice for an assay system because (i) both monomeric and aggregated substrates can be formed depending on the fatty acyl chain lengths, (ii) micelles have no sidedness as do vesicles, and (iii) the individual lecithin molecules have many of the same conformational, dynamic, and packing features of naturally occurring lecithins in bilayers (Burns & Roberts, 1980; Burns et al., 1983; Lin et al., 1986, 1987a). The detailed structures of several short-chain lecithins have been elucidated with small-angle neutron scattering (SANS)[1] (Lin et al., 1986, 1987a,b). Dihexanoylphosphatidylcholine (diC_6PC)[1] forms nearly spherical micelles that do not grow in length with increasing concentration. The fatty acyl chains in these small micelles form a highly disordered core structure (Lin et al., 1986). For a species with more than 12 carbons in the fatty acyl chains, rod-shaped micelles (spherocylinders) are formed that grow in the longitudinal direction with increasing lipid concentration. A series of asymmetric PC's could be fit to spherocylinder models with the same radial structure but different lengths; their growth characteristics (i.e., the length of the rod) can be predicted by a simple thermodynamic model of micelle growth.

Short-chain lecithin substrate analogues have played an important role in determining the structural features of the substrate critical to the activity of phospholipases (Roberts, 1991; Bonsen et al., 1972). The linear symmetric species were used to show that phospholipases preferentially hydrolyze substrate in an aggregated form, i.e., micellar PC is a better substrate than monomeric PC (Roholt & Schlamowitz, 1961; Wells, 1972). In addition to the aggregation state of the lipid (monomer, micelle, or vesicle), chain packing and substrate geometry are factors that also can affect phospholipase activity to some degree. This can often be probed by modifications of the substrate molecule as emphasized by Bonsen et al. (1972) where phospholipids with different backbone configurations, headgroups, and some chain modifications were examined as substrates for phospholipase A_2. For phospholipase C, short-chain ether-linked lecithins were shown to be poor substrates for the enzyme, even though the substrate

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[1] Abbreviations: diC_nPC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; 1-C_n-2-C_m-PC, 1-(*n*)acyl-2-(*m*)acyl-PC; CMC, critical micelle concentration; SANS, small-angle neutron scattering.

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