Engineered plant phosphorylase showing extraordinarily high affinity for various α -glucan molecules

HIROYUKI MORI, KATSUYUKI TANIZAWA, AND TOSHIO FUKUI

Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567, Japan

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Abstract

α -Glucan phosphorylases are characterized by considerable difference in substrate specificities, even though the primary structures are well conserved among the enzymes from microorganisms, plants, and animals. The higher plant phosphorylase isozyme designated as type L exhibits low affinity for a large, highly branched glucan (glycogen), presumably due to steric hindrance caused by a unique 78-residue insertion located beside the mouth of the active-site cleft, whereas another isozyme without the insertion (designated as type H) shows very high affinity for both linear and branched glucans. Using the recombinant type L isozyme from potato tuber as a starting framework and aiming at altering its substrate specificity, we have genetically engineered the 78-residue insertion and its flanking regions. Firstly, removal of the insertion and connection of the newly formed C- and N-terminals yielded a totally inactive enzyme, although the protein was produced in *Escherichia coli* cells in a soluble form. Secondly, a chimeric phosphorylase, in which the 78-residue insertion and its flanking regions are replaced by the corresponding region of the type H isozyme, has been shown to exhibit high affinity for branched glucans (Mori, H., Tanizawa, K., & Fukui, T., 1993, *J. Biol. Chem.* 268, 5574–5581), but when two and four unconserved residues in the N-terminal flanking region of the chimeric phosphorylase were mutated back to those of the type L isozyme, the resulting mutants showed significantly lowered affinity for substrates. Finally, a chimeric phosphorylase, in which a 112-residue sequence of the rabbit muscle enzyme involving the glycogen-storage site was substituted for the 78-residue insertion and its flanking regions of the type L isozyme, showed extraordinarily high affinity for a variety of substrates including not only large, branched glucans but also small, linear ones.

Keywords: α -glucan phosphorylase; glycogen-storage site; protein engineering; substrate affinity

α -Glucan phosphorylase (α -1,4-D-glucan:orthophosphate α -D-glucosyltransferase, EC 2.4.1.1) catalyzes the phosphorysis of various α -1,4-glucan molecules, liberating an energy-conserving sugar phosphate, α -D-glucose-1-phosphate: $(\alpha$ -1,4-glucoside)_n + P_i $\rightleftharpoons$ (α -1,4-glucoside)_{n-1} + α -D-glucose-1-phosphate. The enzyme occurs ubiquitously in nature and has been purified from a wide variety of organisms, including bacteria, fungi, yeasts, slime molds, plants, insects, and mammalian tissues such as skeletal muscle, liver, and brain (Graves & Wang, 1972). The substrate α -glucan molecules can be classified into four groups on the basis of their structures: highly branched with short outer chains (represented by glycogen), moderately branched with long outer chains (amylopectin), long linear (amylose), and short linear (maltodextrin). Al-

though the primary structures of phosphorylases are well conserved among the enzymes from microorganisms, plants, and animals, the substrate specificities for these glucans differ considerably depending on enzyme sources (Newgard et al., 1989). For example, the rabbit muscle enzyme shows high affinity for glycogen but low affinity for amylose and maltodextrin.

On the other hand, higher plant tissues contain two phosphorylase isozymes that are different from each other in molecular size, subcellular localization, and substrate specificity (Fukui et al., 1987). One isozyme designated as type L prefers maltodextrin, amylose, and amylopectin to glycogen as substrate, whereas another isozyme designated as type H exhibits very high affinity for various glucans including glycogen and maltodextrin. The two plant phosphorylase isozymes are encoded by distinct nuclear genes transcribed into mRNAs specific for each isozyme (Nakano et al., 1989; Mori et al., 1991). However,

Reprint requests to: Toshio Fukui, ISIR, Osaka University, Ibaraki, Osaka 567, Japan.

amino acid sequences of the two isozymes show considerably high similarities (>60% identity) with most of the active-site residues being conserved (Mori et al., 1991), except for a lengthy insertion consisting of 78 amino acid residues that occurs in the middle of the polypeptide chain of only the type L isozyme (Nakano & Fukui, 1986). Thus, the low affinity for glycogen of the type L isozyme can be explained, at least partly, by the presence of the 78-residue insertion, which is located beside the mouth of the active-site cleft and presumably exerts steric hindrance for large, highly branched glucans approaching the active site (Nakano & Fukui, 1986).

To elucidate the functional role of the 78-residue insertion uniquely occurring in the plant type L isozyme, we have replaced the insertion and its flanking regions genetically by the corresponding sequence of the type H isozyme, which lacks the insertion. We have also expressed the chimeric enzyme in *Escherichia coli* cells using expression systems constructed for the two plant phosphorylase isozyme cDNAs (Mori et al., 1993). Although the chimeric enzyme was found to be five times less active than the parent type L isozyme, its affinity for glycogen was much higher than that of the type L isozyme and only slightly lower than that of the type H isozyme. The increase in affinity for branched glucans, however, appeared to be more than anticipated based on removal of the 78-residue insertion alone from the type L isozyme; the increase in affinity might also be due to the introduction of a segment from the type H isozyme (Mori et al., 1993). Thus, to explore the high-affinity site in the type H isozyme, we have mutated two and four unconserved residues in the substituted region of the above constructed chimeric enzyme back into those of the type L isozyme. We have also constructed a mutant of the type L isozyme lacking only the insertion and showed that the removal of the insertion without its flanking regions leads to complete loss of catalytic activity. In addition, we have made another chimeric enzyme, in which the 78-residue insertion and the flanking regions of the type L isozyme are replaced by a 112-residue sequence of the rabbit muscle enzyme containing the glycogen-storage site (a preferential glycogen-binding site apart from the active-site region; Kasvinsky et al., 1978). In this paper, we report the results of those studies with particular emphasis on the extraordinarily high affinity of the plant-animal chimeric enzyme for a variety of substrates including not only large, branched glucans but also small, linear ones.

Results

A plant type L phosphorylase mutant lacking the 78-residue insertion

An expression plasmid for the type L phosphorylase isozyme lacking the 78-residue insertion, pELΔIns(78R), was constructed from the type L isozyme gene by loop-

out mutagenesis as outlined in Figure 1A. The encoded polypeptide (GL-78R) is expected to have an amino acid sequence 1–413 plus 492–916 of the type L isozyme, connected at the position of the insertion (Fig. 2) and preceded by a 15-residue peptide derived from the gene 10 protein in the pET vector (Mori et al., 1993).

An immunoblot of the cell extract of *E. coli* carrying pELΔIns(78R) with the anti-type L isozyme rabbit antiserum shows a single band of about 94,000 Da (data not shown), indicating that the type L isozyme mutant lacking the insertion (GL-78R) was produced in a soluble form. However, the cell extract showed no phosphorylase activity when examined by polyacrylamide gel electrophoresis followed by sensitive activity staining (Fig. 3B, lane 6). These results suggest that the removal of the insertion alone brought about steric distortion at the active site, although the whole polypeptide folded into a soluble conformation. The interaction between the 78-residue insertion and the basal part (flanking regions) of the type L isozyme molecule is probably necessary for maintaining the conformation of the active-site structure.

Mutation of N-terminal flanking residues in the chimeric phosphorylase consisting of type L and H isozymes

The recently constructed chimeric phosphorylase (designated hereafter as GL-H; see Fig. 2), in which the 78-residue insertion and its flanking regions of the type L isozyme are replaced by the corresponding sequence of the type H isozyme lacking the insertion, is catalytically active and shows much higher affinity for large, branched glucans than the parent type L isozyme (Mori et al., 1993). To examine whether or not the introduced segments (flanking regions) of the type H isozyme are involved in elevating the affinity for substrate, two (Thr 368 → Gln/Ile 371 → Val) and four (Gln 388 → Tyr/Ala 389 → Glu/Val 390 → Leu/Trp 392 → Gln) unconserved residues in the N-terminal flanking region of the chimeric enzyme were mutated back to those of the type L isozyme, since the N-terminal flanking region is more highly conserved than the C-terminal flanking region (see Fig. 5 in Mori et al., 1993). The constructed plasmids, designated as pELΔIns(M1) and pELΔIns(M2) for the doubly and quadruply mutated genes, respectively, encode the chimeric phosphorylase mutants, designated as GL-H(M1) and GL-H(M2), respectively (Fig. 2).

The *E. coli* BL21(DE3) cells transformed with pELΔIns(M1) or pELΔIns(M2) produced active phosphorylase upon low-temperature (22 °C) cultivation, as observed with the transformant carrying pEL1 (encoding the type L isozyme), pEH3 (encoding the type H isozyme), or pELΔIns (encoding the chimeric enzyme GL-H) (Mori et al., 1991, 1993). The two mutants of the chimeric enzyme were expressed at a level of only about 1% of the total soluble protein but were purified readily by affin-

Fig. 1. Construction schemes for bacterial expression plasmids of the plant type L phosphorylase isozyme lacking the 78-residue insertion (**A**) and the plant-animal chimeric phosphorylase (**B**). Only relevant restriction sites are indicated, and lengths of DNA shown are arbitrary. The restriction fragments are shown by bars and marked differently for an easier understanding of the cloning procedure; lines are the regions from vector DNA. PCR, polymerase chain reaction; RMP, rabbit muscle phosphorylase cDNA. See text for details. **C:** Sequences of the synthetic primers (A4 and A5) for PCR done in procedure B above, aligned with the nucleotide and amino acid sequences of the corresponding regions in both rabbit muscle (template) and type L phosphorylases. Numbers denote the amino acid residue numbers in each enzyme. Restriction sites are underlined, and mismatched bases are shown by asterisks.

Fig. 2. Schematic diagram of the construction strategies for the mutant and chimeric phosphorylases. Only relevant regions for each enzyme are shown by bars in arbitrary sizes and marked differently for easier understanding of the procedures. Numbers denote the residue numbers in the respective sequences. GL-78R, the inactive type L mutant lacks only the 78-residue insertion.

ity chromatography on a glycogen-Sepharose column based on their high affinity for glycogen (see below). The purified enzymes were homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3A, lanes 2 and 3), giving a single band of about 95,000 Da. The specific activities of the purified GL-H(M1) and GL-H(M2) were 3.4 and 7.5 units/mg, respectively, under standard assay conditions, which are about one-tenth and one-fifth, respectively, of that of the parent type L isozyme (Mori et al., 1993).

A chimeric phosphorylase consisting of the plant type L isozyme and the rabbit muscle enzyme with the glycogen-storage site

The glycogen-storage site in the rabbit muscle phosphorylase shows much higher affinity for glucans than the

active site itself (Kasvinsky et al., 1978), serving as an anchoring point through which the enzyme dimer binds to a glycogen particle. The site is composed of a 56-residue subdomain (residues 383–438) and is located in the region corresponding to those flanking the 78-residue insertion in the plant type L isozyme sequence (Nakano & Fukui, 1986; Mori et al., 1993). To engineer the plant type L isozyme toward further improvement of substrate-binding properties, a segment of the rabbit muscle phosphorylase containing the glycogen-storage site was substituted for the 78-residue insertion and its flanking regions in the type L isozyme. An ~330-bp fragment from the rabbit muscle phosphorylase cDNA (Nakano et al., 1986) encoding residues 363–474 of the animal enzyme involving the glycogen-storage site was inserted between *Hind*III and *Sac*I sites occurring in the type L isozyme cDNA (Fig. 1B). The plasmid pELΔIns(RM) thus constructed encodes the

Fig. 3. Polyacrylamide gel electrophoresis of the recombinant phosphorylases. **A:** The purified chimeric enzymes (1 µg each), GL-H (lane 1), GL-H(M1) (lane 2), GL-H(M2) (lane 3), GL-RM (lane 4), and the purified type H isozyme (1 µg; lane 5) were electrophoresed in a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and stained with Coomassie brilliant blue. **B:** The chimeric enzymes (amounts and lane numbers as in A), the type H isozyme (lane 5), and a crude bacterial cell extract (total protein, ~10 µg) expressing the type L isozyme mutant lacking the 78-residue insertion (GL-78R) (lane 6) were electrophoresed in a 5% polyacrylamide gel without SDS and stained for enzyme activity in KI-I₂ solution. **C:** The gel was supplemented with 100 µg/mL dextrin. Other conditions are the same as in B.

plant-animal chimeric phosphorylase (GL-RM) consisting mainly of the plant type L isozyme with 112 residues from the rabbit muscle enzyme in the middle of the polypeptide chain (Fig. 2). The chimeric enzyme GL-RM, expressed in *E. coli* BL21(DE3) cells carrying pELΔIns(RM) and purified to homogeneity (Fig. 3A, lane 4), showed a specific activity of 26.5 units/mg, which is comparable with that (28.7 units/mg) of the recombinant type L isozyme (Mori et al., 1993).

Steady-state kinetic parameters

Apparent steady-state kinetic parameters were determined in both synthesis and phosphorolysis reactions with the three recombinant chimeric enzymes purified, using a fixed and saturating concentration of one substrate in the initial velocity measurements: at 10 mg/mL soluble starch in determination of K_m values for glucose-1-phosphate in the synthesis, and at 60 mM sodium phosphate in determination of K_m values for maltopentaose in the phosphorolysis. In addition, because significant substrate inhibition by maltopentaose at a concentration above 3 mM was observed in the phosphorolysis by all three chimeric enzymes (data not shown), as was seen with the previous chimeric phosphorylase GL-H (pELΔIns) (Mori et al., 1993), K_m values for P_i were determined at 2 mM maltopentaose. The K_m and V_{max} values thus obtained are summarized in Table 1 with standard errors in the least-squares best fit of the experimental data. For comparison, the values previously determined for the rabbit muscle, plant type L and H isozymes, and the chimeric GL-H enzymes (Mori et al., 1993) are also included in Table 1. The plant-animal chimeric enzyme (GL-RM) had a very high V_{max} value, comparable to that of the parent type L isozyme in glucan synthesis, though less than half in the phosphorolysis. On the other hand, the two chimeric phosphorylase mutants, GL-H(M1) and GL-H(M2), had V_{max} values 10 and 5 times, respectively, smaller than that of the parent type

L isozyme in glucan synthesis, as had the chimeric enzyme GL-H (Mori et al., 1993). In contrast, all three chimeric enzymes showed K_m values for maltopentaose similar to that of the type L isozyme, and their K_m values for P_i and glucose-1-phosphate, except for the K_m for P_i of GL-H(M1), were also comparable to those of the type L isozyme.

The kinetic parameters in the phosphorolysis of various other branched and linear glucans were also determined as summarized in Table 2. The three chimeric enzymes, GL-H(M1), GL-H(M2), and GL-RM, had K_m values for branched glucans (glycogen and amylopectin) much smaller than those of the parent type L isozyme, and in particular the K_m value for glycogen of GL-RM was even smaller than those of the rabbit muscle and plant type H enzymes, both of which have rather high affinity for branched glucans. Compared with the previous chimeric enzyme GL-H, both mutants GL-H(M1) and GL-H(M2) had increased K_m values for branched glucans. In addition, a notable substrate inhibition by amylopectin at concentrations above 100 μ g/mL was observed with GL-H(M1) (data not shown). On the other hand, the K_m values of GL-H(M1), GL-H(M2), and GL-RM for linear glucans (amylose DP30 and DP19) were smaller than those of any one of the type L and H isozymes and the rabbit enzyme. As for V_{max} values, all four chimeric enzymes, like both plant isozymes, were more active for linear glucans as substrate than for branched glucans, whereas the rabbit muscle enzyme had almost invariable reactivity for branched and linear substrates.

Dissociation constants

Affinities of phosphorylase for various glucans can be roughly estimated from electrophoretic mobilities of the enzyme in polyacrylamide gels containing immobilized glucans (Shimomura & Fukui, 1980). Typical results of affinity electrophoresis in the presence of 100 μ g/mL gly-

Table 1. Steady-state kinetic parameters

Enzyme	V_{max} (units/mg)		K_m (mM)		
	Synthesis	Phosphorolysis	Glucose-1-phosphate	P_i	Maltopentaose
Type H	91.6 $\pm$ 7.9	33.8 $\pm$ 4.8	5.41 $\pm$ 0.75	1.41 $\pm$ 0.08	1.12 $\pm$ 0.19
GL-H ^a	7.7 $\pm$ 0.6	3.6 $\pm$ 0.3	2.09 $\pm$ 0.27	0.76 $\pm$ 0.09	0.04 $\pm$ 0.01
GL-H(M1)	3.6 $\pm$ 0.1	1.2 $\pm$ 0.2	1.00 $\pm$ 0.05	6.3 $\pm$ 0.8	0.08 $\pm$ 0.02
GL-H(M2)	8.1 $\pm$ 0.2	3.9 $\pm$ 0.5	1.71 $\pm$ 0.10	1.48 $\pm$ 0.16	0.07 $\pm$ 0.02
Type L	39.6 $\pm$ 7.4	16.5 $\pm$ 1.9	2.95 $\pm$ 0.09	1.41 $\pm$ 0.08	0.13 $\pm$ 0.02
GL-RM	29.8 $\pm$ 0.5	7.0 $\pm$ 0.6	2.97 $\pm$ 0.08	0.78 $\pm$ 0.09	0.14 $\pm$ 0.02
Rabbit ^a	ND ^b	26.3 $\pm$ 3.6	ND	ND	16.4 $\pm$ 3.3

^a The kinetic data were taken from Mori et al. (1993).

^b ND, not determined.

Table 2. Kinetic parameters for various glucans in the phosphorolysis

Enzyme	Glycogen		Amylopectin		Amylose DP 30		Amylose DP 19	
	$V_{\max}$ (units/mg)	K_m (μ g/mL)	$V_{\max}$ (units/mg)	K_m (μ g/mL)	$V_{\max}$ (units/mg)	K_m (μ M)	$V_{\max}$ (units/mg)	K_m (μ M)
Type H	9.4 $\pm$ 1.2	9.8 $\pm$ 0.6	8.3 $\pm$ 0.3	3.6 $\pm$ 0.7	18.2 $\pm$ 1.3	8.7 $\pm$ 0.8	9.8 $\pm$ 2.0	22.7 $\pm$ 5.8
GL-H ^a	0.56 $\pm$ 0.06	23.8 $\pm$ 3.6	0.77 $\pm$ 0.06	5.3 $\pm$ 0.7	2.1 $\pm$ 0.02	1.97 $\pm$ 0.03	2.70 $\pm$ 0.01	5.5 $\pm$ 0.4
GL-H(M1)	0.38 $\pm$ 0.06	138.4 $\pm$ 33.2	0.28 $\pm$ 0.03	18.9 $\pm$ 3.3	1.0 $\pm$ 0.08	4.05 $\pm$ 0.49	1.31 $\pm$ 0.06	5.9 $\pm$ 0.5
GL-H(M2)	0.52 $\pm$ 0.04	52.6 $\pm$ 6.3	0.84 $\pm$ 0.21	10.9 $\pm$ 3.5	3.2 $\pm$ 0.41	3.16 $\pm$ 0.61	9.14 $\pm$ 1.3	8.3 $\pm$ 1.7
Type L	8.3 $\pm$ 1.4	10,400 $\pm$ 2,300	7.9 $\pm$ 1.3	82 $\pm$ 17	13.9 $\pm$ 1.5	7.6 $\pm$ 1.0	14.6 $\pm$ 2.0	18.0 $\pm$ 2.9
GL-RM	1.2 $\pm$ 0.1	6.2 $\pm$ 1.6	1.7 $\pm$ 0.7	4.3 $\pm$ 0.7	3.9 $\pm$ 0.5	3.9 $\pm$ 0.8	4.8 $\pm$ 0.5	14.2 $\pm$ 2.2
Rabbit ^a	25.1 $\pm$ 2.9	66.0 $\pm$ 10	22.1 $\pm$ 6.1	310 $\pm$ 60	20.8 $\pm$ 0.7	96.3 $\pm$ 10.2	ND ^b	ND

^a The kinetic data were taken from Mori et al. (1993).^b ND, not determined.

cogen with various recombinant phosphorylases prepared in the present and previous (Mori et al., 1991, 1993) studies are shown in Figure 3C. Because reciprocal plots of the measured mobilities of the three chimeric enzymes against concentrations of amylopectin or glycogen yielded straight lines (data not shown), apparent dissociation constants for glycogen and amylopectin were calculated from the slopes in these plots (Table 3). Although the K_d values obtained did not perfectly coincide with the K_m values (cf. Table 2), it is evident that the three chimeric enzymes have much higher affinity for both glycogen and amylopectin than the parent type L isozyme. In particular, the K_d value for glycogen of GL-RM was smallest even compared with the type H isozyme and the rabbit enzyme, both acting on glycogen as the most preferred substrate. The K_i values for a mobile ligand, maltopentaose, competing with the immobilized ligand when added to the system, were also determined from the electrophoretic mobilities of the enzyme in the presence of a series of fixed concentrations of maltopentaose at various concentrations of an immobilized ligand, amylopectin (Mori

et al., 1993). The K_i values obtained (Table 3) also did not correspond with the kinetic K_m values for maltopentaose (Table 1); the rabbit muscle enzyme as well as the type H isozyme had K_i values much smaller than their K_m values, indicating that the K_i for maltopentaose represents competition with glycogen binding at the storage site. Conversely, the type L enzyme and all the chimeric enzymes had K_i values considerably larger than the K_m values, which probably reflects the high affinity of the active site of the type L isozyme for maltopentaose.

Discussion

The 78-residue insertion uniquely occurring in the plant type L phosphorylase isozyme is located on the surface of the protein and beside the mouth of the active-site cleft (Nakano & Fukui, 1986; Willnecker et al., 1991). This location can explain, even if not completely, the low affinity of the type L isozyme for fairly large, branched glucans such as glycogen and amylopectin in terms of steric hindrance. In fact, the chimeric phosphorylase of the plant type L and H isozymes (GL-H) had affinity for branched glucans much higher than that of the type L isozyme and only slightly lower than that of the type H isozyme, although it was five times less active than the parent enzyme (Mori et al., 1993). Therefore, we conclude that the 78-residue insertion in the type L isozyme is directly related to modulating the affinity for branched glucans. However, a possibility that the excessive increase in affinity of the chimeric enzyme may also be due to the introduction of the type H isozyme segments substituting for the flanking regions of the insertion cannot be ruled out on the basis of the previous results. Thus, to see the effect of removal of the 78-residue insertion alone on the substrate specificity, we have constructed the type L isozyme mutant lacking the insertion and expressed it in *E. coli* cells. The mutant enzyme was indeed expressed in

Table 3. Apparent dissociation constants for glucans determined by affinity electrophoresis

Enzyme	K_d (μ g/mL)		K_i (mM): Maltopentaose
	Glycogen	Amylopectin	
Type H	5.6 $\pm$ 1.2	1.3 $\pm$ 0.3	0.033 $\pm$ 0.008
GL-H ^a	79 $\pm$ 6	7.5 $\pm$ 1.4	0.054 $\pm$ 0.015
GL-H(M1)	69.5 $\pm$ 9.5	4.6 $\pm$ 0.89	0.145 $\pm$ 0.007
GL-H(M2)	131 $\pm$ 30	14.3 $\pm$ 2.3	0.52 $\pm$ 0.10
Type L	52,000 $\pm$ 4,000	1,850 $\pm$ 140	2.9 $\pm$ 0.7
GL-RM	0.81 $\pm$ 0.06	1.6 $\pm$ 0.1	1.38 $\pm$ 0.18
Rabbit ^a	75 $\pm$ 13	15 $\pm$ 2	0.94 $\pm$ 0.23

^a The data were taken from Mori et al. (1993).

E. coli but had no enzymic activity, suggesting that the sole removal of the insertion from the type L isozyme affects deleteriously the structure of the active-site region located about 30 Å apart from the insertion. The results may also indicate that the 78-residue insertion itself does not hold into a domain structure, as it is invisible in the crystal structure of the potato enzyme (Willnecker et al., 1991), but strongly interacts with its flanking regions, which constitute a part of the mouth of the active-site cleft.

The affinity of the phosphorylase active site for the nonreducing end of a glucan substrate is higher in the type L isozyme than in the type H isozyme, as shown by K_m values for maltopentaose (Table 1). Because the type H isozyme has very high affinity for large, branched glucans, it must therefore have another high-affinity site(s) different from the active site and involved in binding of branched glucans (Shimomura et al., 1982). To identify the high-affinity site in the type H isozyme-derived segments (flanking regions) of the chimeric enzyme (GL-H), we have constructed two mutants of GL-H, in which two and four unconserved residues, respectively, were mutated back to those of the type L isozyme (Thr 368 → Gln/Ile 371 → Val in GL-H(M1) and Gln 388 → Tyr/Ala 389 → Glu/Val 390 → Leu/Trp 392 → Gln in GL-H(M2)). The K_m (Table 2) and K_d (Table 3) values for glycogen of GL-H(M1) and GL-H(M2) increased significantly as compared with those of GL-H, while the K_m values for maltopentaose (Table 1) were essentially unchanged. The type H isozyme, like the rabbit muscle enzyme, had K_i values for maltopentaose (Table 3) much smaller than their K_m values (Table 1), whereas the two mutants GL-H(M1) and GL-H(M2) showed increased K_i values. These results strongly suggest that a high-affinity site(s) distinct from the active site is present in the type H isozyme-derived segments in the GL-H chimeric enzyme including these two and four residues. According to the X-ray crystallographic structure of the rabbit muscle enzyme (Acharya et al., 1991), the two residues corresponding to Val 368 and Cys 371 in the animal enzyme are positioned in α -helix 10 and β -sheet 13, respectively, both of which form a junction of the catalytic domain and the subdomain including the glycogen-storage site. The four residues corresponding to Val 388, His 389, Leu 390, and Glu 392 are all included in α -helix 11, which constitutes a part of the glycogen-storage site. Therefore, the high-affinity site in the plant type H isozyme is located presumably in or around the region corresponding to the glycogen-storage site in the animal enzyme. The larger K_m values than the K_d values for branched glucans (glycogen and amylopectin) of GL-H(M1) as well as its high K_m for P_i and the notable inhibition by amylopectin may be due to a tight but disadvantageous mode of glucan binding to the putative high-affinity site of GL-H(M1), leading to the lowered local concentration of the nonreducing ends of substrate at the active site.

The plant-animal chimeric phosphorylase last prepared (GL-RM) showed extraordinarily high affinity for a variety of substrates including not only large, highly branched glucans but also small, linear maltodextrins. This is the first successful example in engineering of phosphorylase toward improvement of the substrate-binding properties. In GL-RM, the markedly increased affinity for large, branched substrates is provided from the introduced glycogen-storage site of the animal enzyme and from the removal of the steric hindrance due to the 78-residue insertion of the parent type L isozyme, while the high affinity for linear glucans afforded by the active site of the type L isozyme is maintained. We do not know why the affinity of the chimeric enzyme for branched glucans is even higher than those of the type H and rabbit enzymes, although the cooperative effect between the active site and the introduced glycogen-storage site may be suggested.

In addition, the considerably decreased V_{max} values of GL-RM particularly in the phosphorolysis may be due to unfavorable conformational changes caused upon binding of glucans to the storage site. In the animal enzyme, it is well known that the activation mechanism is associated with a shift in equilibrium from the low-affinity T-state conformation to the high-affinity R-state (Monod et al., 1965) through tertiary and quaternary structure changes promoted by ligand binding (Barford & Johnson, 1989). A possible pathway for the transmission of conformational changes between the active site and the storage site also has been suggested (Johnson et al., 1990). On the other hand, the plant enzyme (type L isozyme) constantly is in an active form (Lee, 1960a,b), structurally similar to the R-state of the animal enzyme (Willnecker et al., 1991). Thus, the conformational changes induced upon binding of branched glucans to the introduced glycogen-storage site of the chimeric enzyme may produce, in this case, structural distortion of the active site to lower the catalytic efficiency.

Materials and methods

Construction of a plant type L phosphorylase mutant lacking the 78-residue insertion

Genetic manipulation for preparing a mutant of the plant type L phosphorylase isozyme lacking only the 78-residue insertion was done according to the procedure outlined in Figure 1A. First, the *Nco*I-*Xho*I fragment (about 1.5 kbp) and the *Bgl*II-*Sac*I fragment (about 590 bp) were excised from the type L isozyme cDNA harbored in pUC19 (Mori et al., 1993) and then inserted, respectively, into a pTV119N-derived plasmid carrying an *Xho*I site (Mori et al., 1993) and M13 mp18 at *Bam*HI and *Sac*I sites. Using the latter plasmid as a template, the region encoding the 78-residue insertion was eliminated by loop-out mutagenesis (Kunkel et al., 1987), with a synthetic primer

(A1: 5'-GAGGAGCTGGTACATGAAATTVCAGCTG CAGAAAAAGACATT-3'), which corresponds to nucleotides 1,412–1,432 and 1,667–1,687 in the cDNA sequence of the type L isozyme (Nakano et al., 1989), connected at the position of the insertion (inverted triangle). After confirming the nucleotide sequence of the mutant DNA obtained, the *Hind*III–*Sac*I fragment from the former plasmid was exchanged with the same restriction fragment in the mutant gene. Finally, the whole *Nco*I–*Xho*I fragment (1.5 kbp) was substituted for the same restriction fragment in the expression plasmid pEL1 encoding the plant type L phosphorylase isozyme (Mori et al., 1993), leading to the expression plasmid for the type L phosphorylase mutant lacking the insertion, designated as pELΔIns(78R).

Construction of chimeric phosphorylase mutants consisting of the type H and L isozymes

Two (Thr 368/Ile 371) and four (Gln 388/Ala 389/Val 390/Trp 392) residues derived from the plant type H isozyme in the chimeric phosphorylase GL-H constructed recently (Mori et al., 1993) were mutated back into those (Gln 368/Val 371 and Tyr 388/Glu 389/Leu 390/Gln 392) of the type L isozyme by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) on the *Hind*III–*Sac*I fragment from pEH3 (the expression plasmid for the type H isozyme; Mori et al., 1991) that was amplified in advance by polymerase chain reaction (PCR) (Mori et al., 1993) and subcloned into M13 mp18. The following two oligonucleotides were designed as primers for the double and quadruple mutations, respectively:

A2: 5'-AATATCACTCAAAGGACAGTTGCCTATACG-3'

A3: 5'-AAATGGTCATATGAATTGATGCAGAAGCTCCTT-3'

Primers A2 and A3 correspond to nucleotides 1,093–1,120 and 1,153–1,185, respectively, in the cDNA sequence of the type H isozyme (Mori et al., 1991) with several mismatched bases (indicated by asterisks). After confirming the DNA sequence of the mutant fragments, the *Hind*III–*Sac*I fragment from each mutant plasmid was substituted for the original fragment included in the *Nco*I–*Xho*I region of the type L isozyme cDNA on a pTV119N-derived plasmid as described above. Then, the 1.5-kbp *Nco*I–*Xho*I fragments were swapped for the same restriction fragment in pEL1 to give the final expression plasmids for the chimeric phosphorylase mutants, designated as pELΔIns(M1) and pELΔIns(M2), which encode the chimeric phosphorylase (GL-H) with the type H isozyme-derived two (Thr 368 → Gln/Ile 371 → Val) and four (Gln 388 → Tyr/Ala 389 → Glu/Val 390 → Leu/

Trp 392 → Gln) residues being mutated back to those of the type L isozyme.

Construction of a chimeric phosphorylase consisting of the plant type L isozyme and the glycogen-storage site of the rabbit muscle enzyme

The construction scheme for an expression plasmid of the plant-animal chimeric phosphorylase is depicted in Figure 1B. An ~340-bp *Hind*III–*Sac*I fragment in the rabbit muscle phosphorylase cDNA containing the glycogen-storage site (Nakano et al., 1986) was amplified by PCR to be used for replacing the 78-residue insertion and its flanking regions of the type L isozyme. Two primers (A4 and A5) for PCR were designed, as shown in Figure 1C, to correspond to nucleotides 1,160–1,189 in the rabbit enzyme cDNA and nucleotides 1,479–1,505 in the complementary sequence, respectively, supplemented with a restriction site (*Hind*III or *Sac*I, underlined in Fig. 1C). The amplified *Hind*III–*Sac*I fragment was substituted for the same restriction fragment included in the *Nco*I–*Xho*I region of the type L isozyme cDNA on a pTV119N-derived plasmid as described above. Following nucleotide sequencing of the amplified region, the 1.5-kbp *Nco*I–*Xho*I fragment was substituted for the same restriction fragment in pEL1 to yield the expression plasmid of the plant-animal chimeric phosphorylase (GL-RM), designated as pELΔIns(RM).

Bacterial expression and purification of chimeric enzymes

Cultivation of *E. coli* BL21(DE3) cells transformed with either pEL1, pEH3, pELΔIns, pELΔIns(78R), pELΔIns(M1), pELΔIns(M2), or pELΔIns(RM) and cotransformed with pLysS carrying the gene for T7 lysozyme was carried out under the same conditions as described previously (Mori et al., 1991, 1993), which included precultivation at 37 °C for several hours and induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside and 1 mM pyridoxine hydrochloride followed by further cultivation at 22 °C for 12 h. The cells harvested by centrifugation were disrupted at 4 °C in a French pressure cell. The purification method for the chimeric enzymes was essentially identical with that for the chimeric enzyme of type H and L isozymes (GL-H) reported previously (Mori et al., 1993), which consisted of ammonium sulfate precipitation (70% saturation), DEAE-cellulose ion-exchange chromatography, and affinity chromatography on a glycogen-Sepharose column. The enzyme was thoroughly dialyzed at 4 °C against 60 mM sodium phosphate buffer, pH 6.7, to degrade dextran enzymically and to remove the glucose-1-phosphate formed. An average yield of the purified enzyme was about 1 mg from 4 L of bacterial culture in all cases.

Enzyme and protein assays

Enzyme was assayed routinely at 30 °C by the glucan synthesis reaction in 1.0 mL of solution containing 50 mM sodium maleate buffer, pH 6.0, 1 mM EDTA, 20 mM glucose-1-phosphate, and 10 mg/mL soluble starch. The reaction was started by addition of enzyme and stopped by addition of 0.5 mL of ice-cold 5% (w/v) trichloroacetic acid. The liberated P_i was determined, after centrifugation, as described previously (Mori et al., 1993). The Michaelis constants for glucose-1-phosphate were determined under standard assay conditions with variable concentrations (0.5–10 mM) of glucose-1-phosphate.

For determination of apparent Michaelis constants for glucans in the phosphorylase reaction, the consecutive assay method described previously (Mori et al., 1993) was used with various concentrations of a glucan substrate. The Michaelis constants for P_i were determined also by the consecutive assay method with 0.2–20 mM sodium phosphate, pH 6.7, and 2 mM maltopentaose. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and the experimental data were fitted to the Michaelis–Menten equation by the least-squares method using a computer program.

Protein concentration was determined by a microBiuret method with bovine serum albumin as a standard.

Affinity electrophoresis

Apparent dissociation constants for amylopectin and glycogen were determined by affinity electrophoresis according to the method reported previously (Shimomura & Fukui, 1980; Mori et al., 1993) with 4% polyacrylamide gels prepared in glass tubes (0.5 × 12 cm). The enzyme was electrophoresed in the gels containing various concentrations of those glucans with 30 mM Tris-barbital, pH 7.0, containing 0.1 µg/mL bromophenol blue as a running buffer. The electrophoresis was carried out in a cold room (4 °C) at a constant current (2 mA/tube) for 3 h. Reciprocal mobilities of the enzyme (relative to that of bromophenol blue) were plotted against concentrations of added glucans (glycogen or amylopectin), and the apparent dissociation constant (K_d^{app}) of the complex of phosphorylase and an immobilized ligand was calculated from the slopes in these plots by the least-squares method. In determination of apparent dissociation constants for a mobile ligand (maltopentaose) being bound to the enzyme competitively with an immobilized ligand (amylopectin), maltopentaose was added to all enzyme solutions, running buffers, and gels at the same concentration. Dissociation constants for maltopentaose were obtained from the horizontal intercepts in the replots of the K_d^{app} values for amylopectin against concentrations of maltopentaose (Mori et al., 1993).

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