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Rabbit Muscle Phosphorylase Derivatives with Oligosaccharides Covalently Bound to the Glycogen Storage Site†

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ABSTRACT: Linear maltooligosaccharides, e.g., maltoheptaose or terminal 4-*O*-methylmaltoheptaose, activated by cyanogen bromide, react covalently with rabbit muscle phosphorylases *b* and *a* (EC 2.4.1.1). Site-specific modification prevents further binding to glycogen and shifts the phosphorylase *a* tetramer–dimer equilibrium in favor of the dimer. Use was made of these properties to separate by affinity chromatography and gel filtration phosphorylase *a* dimers with specifically bound oligosaccharide from unspecifically modified products. The phosphorylase *a*–maltoheptaose derivative carries one oligosaccharide residue per monomer and can be distinguished from the native enzyme by its electrophoretic mobility in polyacrylamide gels or by affinity electrophoresis. Phosphorylase *a* preparations with covalently bound maltooligosaccharides are enzymatically active in the presence of a primer and α-D-glucopyranose 1-phosphate (glucose-1-P). Methylation of the nonreducing chain terminus of the bound oligosaccharide has no effect on glycogen synthesis. These findings exclude the participation of bound oligosaccharides in chain elongation. Purified covalent phosphorylase *a*–maltoheptaose complexes are stable dimers. They are no longer activated by glycogen. The properties of covalently modified phosphorylase–oligosaccharides are consistent with and provide direct evidence for the existence of a glycogen storage site in rabbit muscle phosphorylases. Covalent occupation of the storage site renders the affinity of glucose-1-P to phosphorylase *a* independent of modulation by glycogen, supporting the assumption that the glycogen storage site is involved in interactions with the catalytic site.

Crystallographic studies of rabbit muscle phosphorylases *a* and *b* (α-1,4-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) in their T conformational states have localized a binding site for linear maltooligosaccharides some 25–30 Å distant from the catalytic site (Fletterick et al., 1976; Weber et al., 1978; Johnson et al., 1980; Fletterick & Madsen, 1980). It has been suggested that this oligosaccharide binding site provides the means to anchor the enzyme to glycogen particles in the living cell (Meyer et al., 1970; Fletterick et al., 1976) and accordingly was named the glycogen storage site. It was further proposed that dissociation of tetrameric phosphorylase *a* to dimers by glycogen or maltoheptaose (Wang et al., 1965; Metzger et al., 1967) results from binding of the poly- or oligosaccharide to the storage site (Kasvinsky et al., 1978). Finally, so that the high affinity for branched polysaccharides and heterotropic cooperativity could be explained (Kasvinsky et al., 1978), the role of an activator site was ascribed to this crystallographically localized oligosaccharide binding site.

The failure to visualize two oligosaccharide sites crystallographically (Fletterick & Madsen, 1980; Johnson et al., 1980) was rationalized on the basis of kinetic studies with maltoheptaose, which indicated that the glycogen storage site has at least a 20 times greater affinity for oligosaccharides ($K_m \simeq 1$ mM) than the catalytic site (Kasvinsky et al., 1978). It was therefore reasoned that the concentration of maltoheptaose (up to 240 mM) used in crystallographic studies might not have been large enough to saturate the catalytic site. In addition, the active site might not be accessible for oligosaccharides in crystals of phosphorylase *a* in the glucose-inhibited T conformation.

Additional experiments are presented to prove more directly the roles ascribed to the glycogen binding site in phosphorylase. We have attacked the problem by covalently coupling cyanogen bromide activated oligosaccharides to phosphorylase and have studied the properties of the covalent phosphorylase–oligosaccharide derivatives, hoping to clarify two points: (1) to check whether phosphorylase preparations covalently modified at the glycogen storage site with oligosaccharides are catalytically active or not and (2) to check more directly whether structural and functional properties of phosphorylases altered by covalent occupation of the glycogen storage site support the anticipated role of an activator site.

Materials and Methods

Glycogen (oyster, type II) was purchased from Sigma Chemical Co. Molar concentrations of glycogen were calculated by assuming 1 out of every 12 glucose residues is an end group (Hu & Gold, 1975). Maltodextrin 19 obtained from

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Maizena GmbH, Heilbronn, contained 92% oligosaccharides. Amylose was from Serva, Heidelberg. NaB^3H_4, [^{14}C]-glucose-1-P,[1] and C^3H_3I were from Amersham-Buchler. High-performance thin-layer chromatography silica gel 60 plates were purchased from Merck, Darmstadt. Aminohexylcellulose was a gift of Merck, Darmstadt. We are greatly indebted to Boehringer, Mannheim, for generous gifts of maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. These maltooligosaccharides were chromatographically pure (>98%) according to high-pressure liquid chromatography.

Enzymes. Phosphorylase *b* was isolated from frozen rabbit muscle by the procedure of Fischer et al. (1958) and recrystallized at least 2 times. Phosphorylase *a* was prepared from phosphorylase *b* with a purified phosphorylase kinase, ATP, and Mg^{2+} (Fischer et al., 1958). Phosphorylase kinase was a generous gift of Dr. H. P. Jennissen, University of Bochum. Protein concentrations were determined spectrophotometrically with $E^{1\%}_{280nm}$ = 13.2 (Kastenschmidt et al., 1968). Molar concentrations of rabbit muscle phosphorylase *a* were calculated on the basis of M_r 97 412 for the monomer (Titani et al., 1977). Maltodextrin phosphorylase from *Escherichia coli* was purified to homogeneity according to Schächtele et al. (1978). Pullulanase was from Boehringer, Mannheim.

Activity Measurements. Initial reaction rates were determined in the direction of polysaccharide synthesis (Illingworth & Cori, 1953). The assay mixture contained 1% glycogen, 16 mM glucose-1-P, 50 mM glycerophosphate, and 20 mM 2-mercaptoethanol buffer, pH 6.8. AMP (1 mM) was added for assay of phosphorylase *b*. Other conditions are detailed in legends to the tables. Phosphorylase *a* and saccharides were preincubated for dimer formation and activation at 30 °C for 15 min prior to assay. The assay was carried out at 30 °C and stopped after 1–10 min, and P_i was determined (Fiske & SubbaRow, 1925).

Glycogen Activation of Phosphorylase. Activation was carried out according to Wang et al. (1965). Samples of native or modified phosphorylase *a* were diluted to 0.7 mg/mL in 15 mM cysteine–20 mM glycerophosphate buffer, pH 6.8 at 20 °C. Activation was started by addition of 1% (5 mM) glycogen (final concentration). From 1 up to 60 min 0.1-mL aliquots were removed and added at 20 °C to 0.1 mL of the substrate mixture, pH 6.8, containing 32 mM glucose-1-P and 2% glycogen. Inorganic phosphate released was measured after 1 min.

Maltooligosaccharides. A series of homologous linear oligosaccharides was prepared by exhaustive treatment with excess pullulanase of prefractionated commercial maltodextrin, containing oligosaccharides of $4 \leq n \leq 10$ in a 10% aqueous solution at pH 5.0 and 25 °C. The mixture of linear oligosaccharides was size fractionated on Bio-Gel P-2 columns (2.5 × 80 cm) at 60 °C (John et al., 1969). The average chain length was determined by measuring total glucose with the phenol–sulfuric acid method and reducing power by Somogyi's method (Hodge & Hofreiter, 1962). The uniformity of the fractions was further checked by high-performance thin-layer chromatography on silica gel 60 precoated sheets (Nurok & Zdatkis, 1978) with 6:4:3 (v/v) 1-butanol–pyridine–water (French et al., 1950) or 10:6:4 (v/v) 1-butanol–acetic acid–water. Fractions from the Bio-Gel P-2 column varied by not more than the two nearest *n* values. Labeled oligosaccharides were fractionated accordingly.

^{3}H-Labeled Maltooligosaccharides. Oligosaccharides ($4 \leq n \leq 8$) were labeled by reduction with NaB^3H_4 (Abdel-Akher et al., 1951). A solution of 50 μmol of oligosaccharide in 0.5 mL of H_2O was treated first with one-tenth of the calculated amount of cold $NaBH_4$ (1.25 μmol) in order to scavenge impurities. Then 15 mCi of solid NaB^3H_4 (≃10 mCi/μmol) was added. After 30 min, reduction was completed by adding a 3-fold excess of cold $NaBH_4$. After acidification with 0.1 M HCl the mixture was evaporated to dryness in a rotary evaporator. The procedure was repeated until constant specific radioactivity was obtained. The products had no reducing power, but the oligosaccharide alcohols formed by reduction were as effective as phosphorylase substrates as the parental compounds.

[^{14}C]Maltoheptaose. A solution containing 40 μmol of maltohexaose and 10 μmol of [^{14}C]glucose-1-P (150 μCi) in 1.75 mL of H_2O, pH 6.8, was incubated with 1 mg of *E. coli* maltodextrin phosphorylase at 30 °C. After 1, 2, and 3 min 10 μmol of cold glucose-1-P was added. After 6 min the reaction mixture was inactivated at 100 °C. On chromatography on Dowex 1-acetate (0.5 × 10 cm) the neutral fractions contained 70% of the radioactivity. Thin-layer chromatography and radioactivity scanning demonstrated the presence of oligosaccharides from n = 6–12. Oligosaccharide fractions differing by not more than one *n* value were obtained from chromatography on Bio-Gel P-2.

Methyl 4-O-Methylmaltooligosaccharides. C^3H_3-4-*O*-$(C_6H_{10}O_5)_nCH_3$ was prepared from 10 g of amylose (M_r 150 000) according to Wing & BeMiller (1972a,b) with the following modifications: After methanolysis of perbenzylated amylose the product was fractionated on a column (2 × 29 cm) of Bio-Beads S-X1, followed by thin-layer chromatography. Fractions excluding the higher and lower extremes were combined and methylated with C^3H_3I. The specific radioactivity of methylated products was determined after conversion of C^3H_3I to methylpyridinium iodide. The perbenzylated methyl 4-*O*-methylmaltooligosaccharide was dissolved in a dimethylformamide–glacial acetic acid mixture and hydrogenolyzed in the presence of 10% Pd on charcoal. The final product mixture was fractionated on Bio-Gel P-2 in H_2O. The average chain length was calculated from the ratio of glucose content to radioactivity. A fraction containing $n \simeq$ 6.9 was used for enzyme modification.

Cyanogen Bromide Activation of Soluble Maltooligosaccharides and Coupling to Amines. Larson & Mosbach (1974) described cyanogen bromide activation of soluble carbohydrates and subsequent coupling to amino residues with 14% yield. We checked cyanogen bromide activation of maltooligosaccharides and subsequent coupling by using a soluble amine or a macromolecular carrier: For that purpose aliquots of a freshly prepared aqueous solution of cyanogen bromide (500 mM) were added to a solution of maltooctaose (20 mM) in water under stirring. The molar ratio of CNBr to oligosaccharide was varied from 1:1 to 8:1. The pH was adjusted and maintained at 10–11 with 0.5 M NaOH. After 5 min at 20 °C when no more NaOH was consumed, activation was considered completed and the pH was lowered to 7.5 with 0.1 M HCl. The activated sugar was added to glycine (40 mM), aminohexylcellulose (60 mM), or bovine serum albumin (10 mg/mL). Coupling was allowed to proceed from 2 to 24 h at pH 7–8.5 at room temperature. Nonactivated maltooctaose added during the coupling reaction to activated maltooctaose and aminohexylcellulose or bovine serum albumin did not affect the yield of coupling.

[1] Abbreviations: *n*, average chain length of oligosaccharides in terms of glucosyl units; glucose-1-P, α-D-glucopyranose 1-phosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate.

Coupling of Cyanogen Bromide Activated Oligosaccharides to Phosphorylase. The oligosaccharide (20 mM) was reacted with a 1.5–2.5-fold molar excess of CNBr. The activated oligosaccharide was added in a 10-fold molar excess (in terms of phosphorylase monomers) to phosphorylase *b* in 0.2 M NaCl–50 mM glycerophosphate buffer, pH 7.0, or to phosphorylase *a* in 0.1 M NaCl–100 mM *N*-methylmorpholine–formate buffer, pH 7.0. The final concentrations of oligosaccharide and phosphorylase were 1.0 and 0.1 mM in terms of monomers, respectively. The reaction mixture was incubated for 3 h at room temperature; when incubation was carried out for 24 h at 4 °C, similar results were obtained. Binding of oligosaccharides to phosphorylase was calculated from the specific radioactivity incorporated after exhaustive dialysis against 50 mM glycerophosphate–20 mM mercaptoethanol buffer, pH 7.0, with and without 0.1 M NaCl. Incorporation was also checked by measurements of radioactivity in protein precipitates on glass-fiber filters before and after repeated washes with 5% trichloroacetic acid.

Affinity Chromatography. In contrast to *E. coli* phosphorylase, which was readily bound to Sepharose–glycogen (Schächtele et al., 1978), rabbit muscle phosphorylase *a* was not retained unless the enzyme was preincubated with the affinity matrix. In 10 mM Tris buffer, pH 7.0, 1.5 mg of phosphorylase *a* was bound to 1 mL of Sepharose–glycogen after incubation for 30 min at 25 °C.

Affinity chromatography on Sepharose–glycogen was used for the fractionation of phosphorylase preparations with covalently bound oligosaccharides according to their residual affinity for glycogen. For that purpose disposable plastic syringes (2–20 mL), fitted with a porous polyethylene disk (e.g., Amicon membrane support), were packed with the gel suspended in starting buffer. The settled gel was freed of buffer by centrifugation at 2000 rpm or by applying pressure. The removed buffer volume was replaced with 2–4 mg/mL phosphorylase in 1 mM dithioerythritol–10 mM Tris buffer, pH 7.0 (starting buffer). Unbound protein was collected in the eluate by centrifugation after 30 min. Elution was repeated twice with an equal volume of starting buffer and 100 mM NaCl in the buffer. For large-scale preparations 30 × 2 cm Sepharose–glycogen columns were used.

Binding Assay with [^{3}H]Maltoheptaose and Native and Covalently Modified Phosphorylase a. Conventional binding assays using equilibrium dialysis cannot be used because of the low affinity, $K_{diss} > 1$ mM, of phosphorylase for oligosaccharides. An attempt was made, however, to assess binding qualitatively by filtration through nitrocellulose filters (Suter & Rosenbusch, 1976). Phosphorylase *a* (0.1 mM monomer) was equilibrated with [^{3}H]maltoheptaose at concentrations ranging from 0.2 to 2 mM in 10 mM glycerophosphate–10 mM mercaptoethanol buffer, pH 7.0, at room temperature. Aliquots (0.5 mL each) of the reaction mixture were passed through nitrocellulose filters without washing with buffer. Controls were run with [^{3}H]maltoheptaose either with or without bovine serum albumin.

Polyacrylamide Gel Electrophoresis with and without Glycogen (Affinity Electrophoresis). The electrophoresis was carried out in 12 × 10 × 0.15 cm slab gels containing 5.1% acrylamide and 0–0.1% glycogen according to Shimomura & Fukui (1980), except that the pH of the 70 mM Tris-HCl buffer was 8.0 and the running buffer solution was 30 mM barbital–Tris, pH 7.4. The conditions for electrophoresis were 50–70 mV and 18 mA per slab gel at room temperature. The time for electrophoresis corresponded to the time required for a 15-cm movement of the bromphenol blue dye marker. The gels were stained by Coomassie Blue and scanned with a Berthold densitometer.

Ultracentrifugal Analysis. Phosphorylase preparations were analyzed in the analytical Spinco Model E ultracentrifuge according to Seery et al. (1967). Sedimentation velocity runs were made at 44 000 or 60 000 rpm at 20 °C. The buffers used contained 0.1–0.3 M NaCl.

Sucrose Density Gradient Centrifugation (Martin & Ames, 1961). Samples (1–2 mg in 0.25 mL) of phosphorylase preparations were layered on top of 10 mL of a 20–5% sucrose gradient in 50 mM glycerophosphate–50 mM mercaptoethanol buffer, pH 7.0. Centrifugation was for 7–9 h at 40 000 rpm and 20 °C in a SW 41 rotor.

Results

Preparation and Purification of Site Specifically Modified Phosphorylase–Oligosaccharide Derivatives. Affinity labeling was applied in this work to a protein with known primary sequence and three-dimensional structure and an equally well-defined ligand (Titani et al., 1977; Fletterick & Madsen, 1980). The size of the glycogen binding domain is, however, rather large. Moreover, the nature and number of amino acid residues that are available for the reaction with chemically activated glucose units are not known. Therefore, one cannot predict which section of an oligosaccharide chain might react with the binding site. We have therefore used on purpose oligosaccharides with randomly activated glucose units and have devised a separation method for the isolation of correctly derivatized phosphorylase on the basis of expected properties of such derivatives. Cyanogen bromide activation of soluble oligosaccharides was used because a rather uniform distribution of activated glucose residues can be obtained with this procedure (Larsson & Mosbach, 1974). Moreover, this procedure has the advantage that phosphorylase attached to a carbohydrate carrier by the cyanogen bromide coupling method was shown to retain catalytic activity (Feldmann et al., 1972; Sotiroudis et al., 1978).

The extent of accessibility and reactivity of cyanogen bromide activated oligosaccharides for low and high molecular weight coupling partners was first evaluated: Maltooctaose activated by a 2–8-fold molar excess of cyanogen bromide reacted with an average of 1.5 molecules of glycine. However, only 1 out of 3–5 molecules of activated maltooctaose was covalently bound to aminohexylcellulose, and 1 out of 7–10 molecules was bound to bovine serum albumin.

In the case of phosphorylase as a coupling partner, several possibilities had to be considered: The activated oligosaccharide might bind preferentially at the glycogen storage site and/or to the active site and/or to other functionally undefined sites. The final reaction conditions were chosen to provide an excess of reactive oligosaccharide at a concentration close to the dissociation constant of the glycogen storage site. The apparent stoichiometry of oligosaccharide binding was calculated from the incorporation of radioactively labeled oligosaccharide derivatives by using different methods for separation of unreacted substrate such as ammonium sulfate precipitation, dialysis, Sephadex G-25 chromatography, and fractional crystallization (Table I).

The initial modification studies were performed with phosphorylase *b*. The molar ratio of oligosaccharides bound per phosphorylase monomer in unfractionated products ranged from 0.5 to 2, depending on conditions. Thus besides unmodified phosphorylase, phosphorylase derivatives labeled at more than one site per monomer were present in the unfractionated preparations. In control experiments, phosphorylase *b* was modified in the presence of an excess of glycogen, which

Table I: Preparation and Properties of Phosphorylase–Oligosaccharide Derivatives[a]

conditions of affinity labeling			molar ratio of oligosaccharides bound per phosphorylase monomer		
enzyme form	ligand[b]	molar ratio, CNBr/oligosaccharide	unfractionated products	after affinity chromatography	after dimer separation
phosphorylase *a*	maltoheptaose	2.0	1.3–1.5	1.0–1.5	1[d]
phosphorylase *a*	maltotetraose	2.0	0.5	0.67	1[d]
phosphorylase *b*	terminal 4-*O*-methylmaltoheptaose	2.5	1.8[c]		~1[d]

[a] Phosphorylase (0.1 mM) was coupled with cyanogen bromide activated oligosaccharides (1 mM) for 2 h at 20 °C. Covalent incorporation of radioactively labeled ligand was determined in the products after ammonium sulfate precipitation and repeated dialysis. The products were further fractionated by exclusion from Sepharose-glycogen affinity chromatography and Sephacryl S-300 chromatography as described. Experiments with maltoheptaose and maltotetraose were repeated 3–6 times. [b] Labeled oligosaccharides were reduced [^{3}H]maltoheptaose, [^{14}C]maltoheptaose, reduced [^{3}H]maltotetraose, and terminal 4-*O*-[^{3}H]methylmaltoheptaose. [c] The phosphorylase *b* derivative was subsequently converted to the *a* form and chromatographed on Bio-Gel P-300. [d] The ratio was obtained from the dimer peak fraction.

Table II: Comparison between Sedimentation of Phosphorylase *a*–Maltoheptaose Derivatives and Phosphorylase *a* with and without Maltoheptaose

enzymes	conditions	$s_{20,w} \times 10^{13}$ (S)
phosphorylase *a*	7.0 mg/mL, 20 °C	13.4
phosphorylase *a*	2.9 mg/mL, 22 °C, 10 mM maltoheptaose	9.7[c]
phosphorylase *a* coupled with maltoheptaose (unfractionated)[a]	5.7 mg/mL, 20 °C	18 (25%) 13.4 (50%) 8.0 (25%)
phosphorylase *a*–maltoheptaose[b]	0.5 mg/mL, 20 °C	8.1 (100%)

[a] Phosphorylase *b* was covalently modified by cyanogen bromide activated maltoheptaose and converted to phosphorylase *a*. [b] Isolated from Bio-Gel P-300 chromatography. [c] From Metzger et al. (1967).

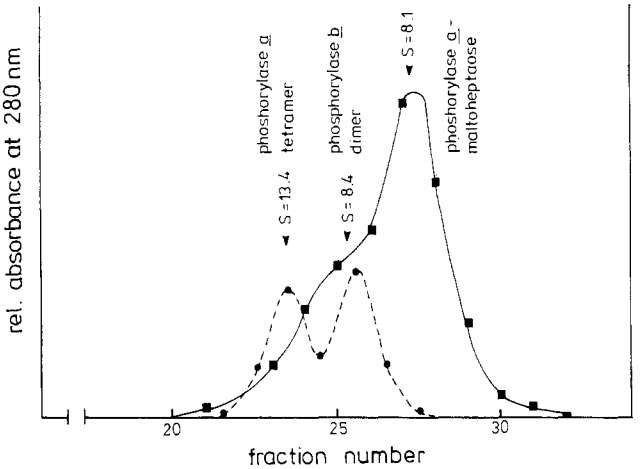

FIGURE 1: Typical elution patterns for phosphorylase–maltoheptaose derivatives and phosphorylases *a* and *b* from Sephacryl S-300. The enzymes were eluted from a 2 × 95 cm column in a 50 mM glycerophosphate, 20 mM mercaptoethanol, 100 mM NaCl buffer, pH 6.8, at 20 °C and a flow rate of 30 mL/h. Fractions of 7.25 mL were collected: (■) phosphorylase *a*–maltoheptaose derivatives collected from the flow through of a Sepharose–glycogen affinity column; (●) phosphorylase *a* and phosphorylase *b* (1:1 mixture). The arrows indicate relative size by their sedimentation constant in the analytical ultracentrifuge.

significantly lowered the molar ratio of covalent incorporation of the activated saccharide. Competition with glycogen had no effect on the coupling reaction with albumin. These findings indicate a preferential binding of the activated oligosaccharides at glycogen binding sites.

Phosphorylase a Dimer Derivatives. It has been reported that millimolar concentrations of maltoheptaose in the absence of second substrates (P_i, glucose-1-P) reversibly dissociate inactive phosphorylase *a* tetramer into active dimers (Wang et al., 1965; Metzger et al., 1967). This observation let us expect to characterize and enrich specifically modified phosphorylase *a* as stable dimers.

Table II compares the sedimentation pattern of a phosphorylase *b* preparation with bound maltoheptaose and converted to phosphorylase *a* with data from Metzer et al. (1967). Approximately 25% of the preparation sedimented as a dimer, the bulk as a tetramer with some higher aggregates. Similar results were obtained by sucrose gradient centrifugation and Sephadex G-200 gel filtration. The dimer fraction obtained after molecular sieving was reanalyzed in the analytical ultracentrifuge. Monodispersity of the solute and sedimentation values characteristic for phosphorylase dimers ($s_{20,w}$ = 8.4 S; Seery et al., 1967) suggest that the dimer population is rather stable and slow reequilibration of oligomers did not take place.

If the modification was carried out with phosphorylase *a* rather than phosphorylase *b*, the amount of dimer formed was greater than 50%. Hence, all subsequent modifications were carried out with phosphorylase *a* (Table I).

Affinity Chromatography. Studying the conditions for affinity chromatography of phosphorylase *a* and its derivatives, we found that the binding of phosphorylase *a* to glycogen, immobilized on Sepharose, is dependent on preincubation with the affinity matrix. This corroborates the findings of Wang et al. (1965) and Metzger et al. (1967) whereby glycogen binds exclusively to phosphorylase *a* dimers that are formed from tetramers in a relatively slow, temperature-dependent dissociation reaction. An affinity-based separation procedure was therefore designed, expecting that phosphorylase *a* dimers not modified at the glycogen storage site should be retained on incubation with Sepharose–glycogen. Modification of phosphorylase at the storage site should, however, exclude or diminish binding; the simultaneous formation of enzyme dimers should provide identification of the expected products. A characterization of the fraction not retained on Sepharose–glycogen by gel filtration on Sephacryl S-300 proved this assumption basically to be correct. As shown in Figure 1 this fraction now contained more than 80% of the phosphorylase–maltoheptaose derivative as a dimer (although modified phosphorylases were retarded slightly when compared with native phosphorylases). The oligosaccharide/monomer ratio (Table I) indicated that molecules with a ratio of 1:1 are selectively enriched in the dimer fraction. The fairly broad shoulder (Figure 1) indicating heavy material generally was of higher specific radioactivity as a result of additional oligosaccharide binding. It was checked for enzymatic activity and found nearly inactive. It was not further analyzed, but it was not contaminated measurably with native unmodified enzyme. Likewise some trailing of the dimer peak was

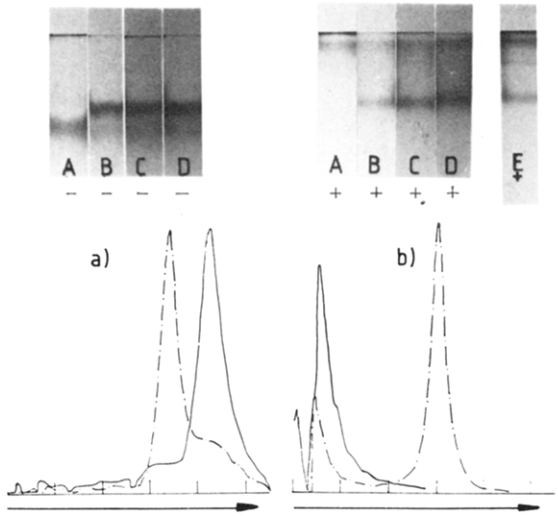

FIGURE 2: Polyacrylamide gel electrophoresis profiles of phosphorylase *a* and phosphorylase *a*–maltoheptaose derivatives in the absence and presence of glycogen. Conditions were as follows: 5.1% polyacrylamide slab gels, 12 × 10 × 0.15 cm, pH 7.4, (–) without glycogen, (+) with 0.1% glycogen. (A) Phosphorylase *a*; (B) supernatant of the dialyzed product mixture after modification of phosphorylase *a* with maltoheptaose; (C) flow through after Sepharose–glycogen affinity chromatography; (D) dimer fraction from Sephacryl S-300 chromatography; (E) phosphorylase *a*–maltoheptaose preparation corresponding to step D but aged for 10 days. (a and b) Densitometric pattern taken from A(–), D(–), A(+), and D(+); one unit on the lower scale corresponds to 0.5 cm in the gel. The percentage of the major band was (A) 93%, (B) 71%, (C) 73%, and (D) 83%.

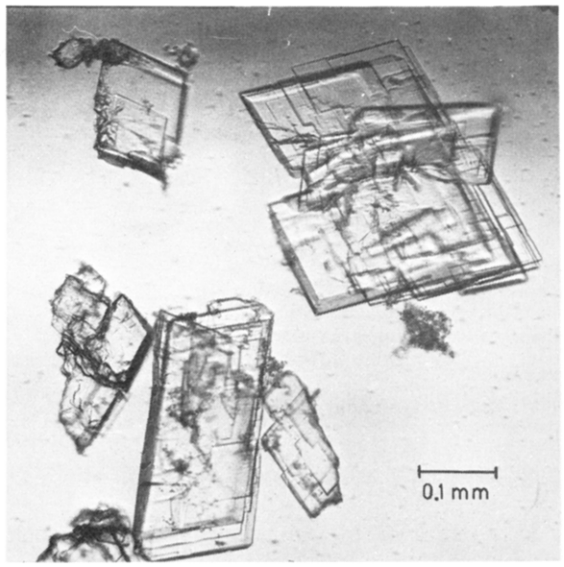

FIGURE 3: Crystals of a phosphorylase *a* preparation containing covalently bound maltoheptaose. Crystallization was obtained from a preparation subjected to affinity chromatography on Sepharose–glycogen and transferred into 10 mM 3-(*N*-morpholino)propanesulfonic acid, 0.1 mM EDTA, 10 mM magnesium acetate, and 3 mM dithioerythritol buffer, pH 6.8. Microcrystals (10 mg/mL) were dissolved at 35–39 °C, centrifuged, and incubated at 32–25 °C.

sometimes observed.

Homogeneity of Phosphorylase–Oligosaccharide Complexes. We have monitored the various stages of the purification procedure (Table I) by polyacrylamide gel electrophoresis in the absence and presence of glycogen (Figure 2a,b). Loss of binding of glycogen in the affinity gel was interpreted to indicate a blocked glycogen storage site. On modification, a major new protein band was found, which is distinguished from native phosphorylase by its electrophoretic mobility and altered binding to glycogen (Figure 2B). The proportion of the major band increased further after affinity chromatography and constitutes 83% after dimer separation (Figure 2C,D). Activity staining of gel rods showed that the enzyme activity was roughly proportional to the protein staining.

The mobility of the minor protein band was affected in the presence of glycogen. While we were not successful in separating this component on a preparative scale, we could show that the minor component was also derivatized with radioactively labeled oligosaccharide but had decreased enzymatic activity. Most probably the minor protein band represents a fraction that is unspecifically covalently modified.

In aged preparations increasing amounts of the enzyme derivative were found to bind to glycogen (Figure 2E). This fraction was lost on repeated cycles of affinity chromatography. As a consequence, only freshly prepared phosphorylase *a* derivatives processed up to the dimer isolation step were used for subsequent kinetic studies. These preparations gave highly reproducible results.

Binding Assays. Filter binding assays (Suter & Rosenbusch, 1976) always indicated that variable amounts of maltoheptaose were bound to native phosphorylase *a* whereas no binding of the oligosaccharide to phosphorylase *a*–maltoheptaose derivatives was observed under any circumstance.

Crystallization of Phosphorylase a–Maltoheptaose Derivatives. Phosphorylase *a* with covalently bound oligosaccharide crystallized readily in the crystallization buffer of Green & Cori (1943) or in buffers of low ionic strength (μ = 0.1). Figure 3 shows crystals of a phosphorylase *a*–maltoheptaose derivative after affinity chromatography on Sepharose–glycogen. The crystals represent a pile of thin plates and are fragile. Crystal suspensions stored for a period of more than 1 month lose about 20% of the radioactive oligosaccharide, but the original ratio of bound oligosaccharide to enzyme is reestablished on repeated affinity chromatography. Crystalline phosphorylase derivatives with covalently bound oligosaccharide might be of interest to the crystallographer.

Distinction between Catalytic Site and Glycogen Storage Site. Covalent modification of a glycogen binding site with the characteristics of a storage or activator site should allow us to decide whether this site participates also in the catalytic reaction, for example, by transfer of polysaccharide chains to the catalytic site. To that end we have bound to the storage site an oligosaccharide with the 4-O position of the nonreducing chain terminus blocked by methylation. Terminal 4-*O*-methylmaltoheptaose did not serve as a substrate for native muscle phosphorylase *b* or *a* but competed for maltooligosaccharides. The K_i value for the 4-*O*-methylmaltoheptaose analogue determined by competitive inhibition with glycogen and phosphorylase *a* was 2.6 mM and similar to the $K_{g'}$ value of 1.0 mM for maltoheptaose determined by Kasvinsky et al. (1978).

The phosphorylase–4-*O*-methylmaltoheptaose derivative (Table I) had properties indistinguishable from those of an unblocked phosphorylase–maltoheptaose derivative, including catalytic reactivity with glucose-1-P and saccharide substrates. This virtually excludes the possibility that a saccharide bound at the storage site can participate in catalysis. Furthermore, our results are in agreement with observations of Sotiroudis et al. (1978), who used phosphorylase *b* covalently bound to high molecular weight glycogen and found no significant differences between native and modified phosphorylase in the capacity to utilize glycogen.

Glycogen Activation. If activity is a function of the concentration of active phosphorylase dimers formed in the

Table III: Activation of Native Phosphorylase *a* and Covalent Phosphorylase *a*-Oligosaccharide Derivatives by Glycogen[a]

enzymes	relative sp act.[c] after 0 min	60 min
phosphorylase *a*	0.11	1
phosphorylase *a*-maltoheptaose[b]	0.83	1
phosphorylase *a*-terminal 4-*O*-methylmaltoheptaose[b]	1.0	1

[a] Activation by glycogen was initiated by addition of 1% (5 mM) glycogen to the enzyme (0.7 mg/mL) preincubated at pH 6.8 and 20 °C. At intervals ranging from 1 to 60 min, aliquots were assayed for enzyme activity at 20 °C (see Materials and Methods). [b] The enzyme derivatives are from the dimer fraction (see Table I, last column). [c] The specific activity obtained after complete activation (60 min) was taken as 1 (100%).

equilibrium reaction as postulated by Metzger et al. (1967), the covalently modified dimer–oligosaccharide derivatives should not only be active but moreover activity should no longer require preincubation with glycogen. This is indeed the case as a comparison of native phosphorylase *a* with modified phosphorylase *a* dimers shows (Table III). The phosphorylase–maltoheptaose complex had 83–100% activity without preincubation with glycogen. Since activation on incubation of phosphorylase *a* tetramers with glycogen is rather slow at 20 °C [see also Wang et al. (1965)], it follows that the modified enzyme dimers are a priori active. Residual activation may be caused by enzyme molecules with a free glycogen storage site.

On the basis of this assumption, one can even calculate the proportion of enzyme species that is still subject to glycogen activation. Thus a 17% residual activation (Table III) would be caused by only 3% of native phosphorylase. However, since the fractional activation was approximately proportional to the minor component observed in electrophoresis, it is more likely that unspecific derivatization accounted for the residual activation.

The response to glycogen provides also a means to estimate to what extent kinetic data might be influenced by the presence of phosphorylase derivatives with properties different from those of the phosphorylase–maltoheptaose derivative. It can be concluded that such hypothetical contaminants may change velocity data by less than 20% at substrate saturation. In regard to affinity, dissociation constants or K_m values will of course always be determined by the more affine component, provided it is present in sufficient amounts.

Kinetic Studies of Modified Phosphorylase Derivatives. Kinetic parameters of native and modified phosphorylase *a* are compared in Table IV. With glycogen as the variable substrate normal linear double-reciprocal plots were obtained. The K_m values for glycogen and the phosphorylase–maltoheptaose derivative were 1.5–2-fold larger than the corresponding values for native phosphorylase. This may be taken as an indication of decreased affinity for glycogen. Hence, the presence of another enzyme species that still retains high affinity of the storage site would be expected rather to decrease than increase the apparent K_m value for glycogen. When linear oligosaccharides like maltoheptaose instead of glycogen were used as variable substrates, modification was without influence on the respective K_m values for maltoheptaose. Reports in the literature vary considerably: 21.7 mM (Kasvinsky et al., 1978), 33 mM (Hu & Gold, 1975), 70 mM (Goldemberg, 1962), and 172 mM (Kasvinsky & Madsen, 1976). We likewise obtained different apparent K_m values for maltoheptaose in the higher and lower concentration range, but the modified and the native enzyme behaved alike (Table IV).

The most pronounced changes in kinetic properties were observed when the activity of modified phosphorylase was studied as a function of glucose-1-P concentration (Figure 4). The K_m values for glucose-1-P at saturating concentrations of glycogen approached a lower limit of about 3 mM for native and modified phosphorylase. At 4–8 mM concentrations of maltoheptaose, which do not fully saturate the storage site, the apparent K_m value for glucose-1-P and native phosphorylase *a* was 3 times larger than in the presence of glycogen, whereas with modified phosphorylase *a* the K_m value for glucose-1-P in the presence of maltoheptaose did not differ much from the corresponding K_m value in the presence of glycogen. A 2.4-fold increase of K_m (G-1-P) values for phosphorylase *a* at low levels of maltoheptaose can also be derived from the plots presented by Kasvinsky et al. (1978). Our data therefore support and prove actually more directly the capability of the glycogen storage site to interact with the catalytic site as was proposed by Kasvinsky et al. In the case

Table IV: Kinetic Parameters for Native Phosphorylase *a* and Covalent Phosphorylase *a*-Oligosaccharide Derivatives[a]

enzyme preparation	variable substrate (mM fixed substrate)	app K_m values (mM)	K_m(mod)/K_m[b]	V (μmol·min^{-1}·mg^{-1})
phosphorylase *a*	glycogen (16 mM glucose-1-P)	0.1–0.14		53
phosphorylase *a*-maltoheptaose	glycogen (16 mM glucose-1-P)	0.2–0.26	1.5–2.0	25
phosphorylase *a*-maltotetraose	glycogen (16 mM glucose-1-P)	0.15	1.1	33
phosphorylase *a*	maltoheptaose[c] (16 mM glucose-1-P)	87 (33)		160 (30)
phosphorylase *a*-maltoheptaose	maltoheptaose[c] (16 mM glucose-1-P)	87 (33)	1.0	23 (7)
phosphorylase *a*	glucose-1-P (5 mM glycogen)	3.3		53
	glucose-1-P (4 mM maltoheptaose)	10.1		3.4
phosphorylase *a*-maltoheptaose	glucose-1-P (5 mM glycogen)	3.1	0.92	32.5
	glucose-1-P (4 mM maltoheptaose)	3.1	0.31	2.1
phosphorylase *a*	glycogen (16 mM glucose-1-P, 5 mM terminal 4-*O*-methylmaltoheptaose)	2.6[d]		

[a] The kinetic experiments were carried out at 30 °C in 50 mM glycerophosphate–20 mM 2-mercaptoethanol or 10 mM glycerophosphate–10 mM magnesium acetate–1 mM EDTA–5 mM dithioerythritol–0.05% bovine serum albumin buffer, pH 6.8. [b] Ratios of K_m values are derived from data obtained from one given enzyme preparation and subsequent modification. [c] Concentration range of maltoheptaose was 12–72 mM (and 3–15 mM; values in parentheses). For details see text. [d] K_i value.

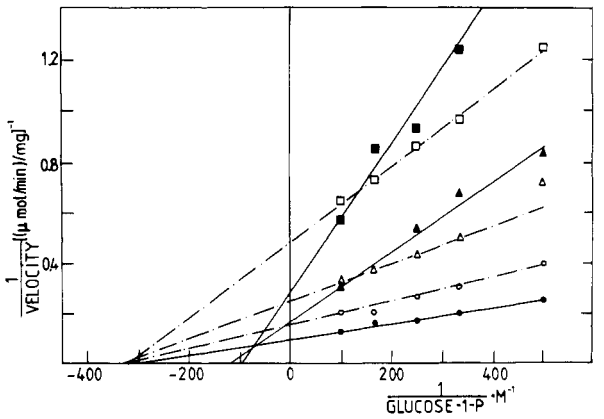

FIGURE 4: Activity of phosphorylase *a* and covalent phosphorylase *a*–maltoheptaose as a function of glucose-1-P. Enzymatic activity of native phosphorylase *a* (filled symbols) or covalent phosphorylase *a*–maltoheptaose (open symbols) was assayed at the concentrations of the second substrate as follows: (■, □) 4 mM maltoheptaose; (▲, △) 8 mM maltoheptaose; (●, ○) 5 mM glycogen (in terms of end groups); 1/velocity data in the presence of glycogen were multiplied by a factor of 5 only for legibility. Lines are drawn by a least-squares fit. Other conditions were as described in Table IV.

of small oligosaccharides with a more limited range for interactions with subsites, the extent of the heterotropic effect would be determined by the extent of saturation of and bond formation with the glycogen storage or activator site. Along these lines one could argue that covalent modification of the activator site with linear oligosaccharides desensitized and froze the enzyme in a conformation that binds glucose-1-P as tightly as native phosphorylase *a* saturated with glycogen. Similar, but smaller, effects observed by Sotiroudis et al. (1978) with phosphorylase *b* covalently bound to glycogen might be explained accordingly.

Discussion

The results of the kinetic experiments need to be discussed further: Kinetic evidence suggested that glucose-1-P binding is promoted by positive cooperative site–site interaction between the glucose-1-P site and the glycogen (oligosaccharide) site (Kasvinsky et al., 1978). Saturating concentrations of glycogen effect a complete shift to a state characterized by tight glucose-1-P binding. The fact that covalent occupation of the glycogen storage site by maltoheptaose permanently locks the enzyme in a tight binding conformation provides direct evidence that the glycogen storage site is indeed responsible for heterotropic site–site interaction in the native enzyme.

The much lower K_m value for glycogen and phosphorylase compared to maltoheptaose was interpreted by Kasvinsky et al. (1978) to result from simultaneous binding of the branched polysaccharide to the catalytic and the glycogen storage site. But, when the glycogen storage site is covalently modified, thus leaving accessible only the catalytic site, one would anticipate similar dissociation constants for branched and linear saccharides in terms of molar concentrations of chain termini. This, however, was not the case. K_m values for glycogen and maltoheptaose and modified phosphorylases still differed by nearly 2 orders of magnitude. As a fact these values were nearly the same for the native and the modified enzyme. It follows that accessibility of the glycogen storage site and its occupancy cannot account quantitatively for the much lower K_m values for branched compared with linear saccharides. A calculation based on published data for semisynthetic branched oligosaccharides (Hu & Gold, 1975) shows a similar discrepancy: In that case too, the 10–20 times higher K_m values for the oligosaccharides cannot be quantitatively accounted for in terms of a highly affine glycogen storage site. In order to reconcile these discrepancies with the kinetic model of Kasvinsky et al., one would have to speculate that in our case the small oligosaccharides used for the modification of the glycogen storage site, despite their proven efficacy with respect to dimer formation and activation, do not occupy the glycogen storage site in a functionally optimal way, assuming that for polysaccharide binding additional interactions with subsites at the storage site are required (Fletterik & Madsen, 1980). This is not likely however, since much higher K_m values for linear oligosaccharides than for glycogen were likewise observed with native phosphorylases. Thus, the assumption that covalent modification of the storage site is destructive and/or incomplete, causing loss of affinity to the catalytic site, does not offer a satisfactory explanation. More likely, the site–site interactions between storage and catalytic sites postulated by Kasvinsky et al. (1978) are permissive rather than obligatory for catalysis. Moreover, a sequential mechanism of binding where binding of poly- and oligosaccharides to a high-affinity storage site is a necessary prerequisite for activity as suggested by Fletterick & Madsen (1980) does not appear to be obligatory for all phosphorylases. More primitive ancestral phosphorylases such as *E. coli* maltodextrin phosphorylase, which handle preferentially linear oligo- and polysaccharides, have only one oligosaccharide binding site (D. Palm, unpublished experiments). The situation for potato phosphorylase seems to be intermediate. The polysaccharide binding site may be divided into two subsites (Shimomura & Fukui, 1980). Thus, the additional glycogen storage or activator site may be an evolutionary advance enabling phosphorylases to handle highly branched α-glucans such as glycogen more effectively.

All the properties of the enriched dimer fraction derived from oligosaccharide–phosphorylase *a* derivatives are compatible with the assumption that the covalently labeled oligosaccharide binding site is identical with the glycogen binding site instrumental in dimer formation. It is therefore of obvious interest to identify a radioactively labeled oligosaccharide containing peptide and its location in the sequence. We have not yet been able to identify and isolate such a peptide after partial digestion with trypsin and other proteases. Incubation of phosphorylase modified with radioactively labeled oligosaccharides in 0.5 M glycine at pH 7.5 led to a loss of 50% label of the bound oligosaccharide in about 1 day. We have assumed that the isourea bond originating from the cyanogen bromide activated glucosyl residues was displaced by amino groups forming a guanidino enzyme, as was shown by Wilchek et al. (1975) to be the case for cyanogen bromide activated Sepharose-bound insulin derivatives. If that is the case, it should be possible to replace the bound oligosaccharide by a guanidino derivative (Tesser et al., 1974) and proceed from here to the isolation, purification, and sequence determination of the glycogen binding peptide. This work is now in progress in collaboration with Dr. Emile Schiltz from this laboratory.

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Specificity of Isolectins of Wheat Germ Agglutinin for Sialyloligosaccharides: A 360-MHz Proton Nuclear Magnetic Resonance Binding Study†

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ABSTRACT: The binding of three purified sialic acid containing oligosaccharides to two isolectins of wheat germ agglutinin (WGA I and WGA II) has been quantitated by measuring the broadening of a ligand resonance in the proton nuclear magnetic resonance (^{1}H NMR) spectrum at 360 MHz. The ligands, isolated from bovine colostrum by using the procedure of Schneir and Rafelson [Schneir, M. L., & Rafelson, M. E., Jr. (1966) *Biochim. Biophys. Acta 130*, 1–11], were identified by ^{1}H NMR as the α(2,3) and α(2,6) isomers of *N*-acetylneuraminyllactose, as well as the α(2,6) form of *N*,*N*′-diacetylneuraminyllactosamine. The dissociation constants, K_D's, ranged from 0.7 to 10 mM (24 ± 1 °C). Two noteworthy features of WGA specificity emerge from an examination of the observed affinities: (1) both isolectins bind the α(2,3) isomer of *N*-acetylneuraminyllactose with higher affinity than the α(2,6) form and (2) WGA I binds two of the sialyloligosaccharides more tightly than does WGA II.

The use of plant lectins as probes of membrane carbohydrate structure is widespread in many areas of biological research [for reviews, see Nicolson (1974) and Goldstein & Hayes (1978)]. It is generally believed that investigations of lectin–sugar interactions will be essential to our understanding of such processes as differentiation, cancer, and metastasis, since these are theorized to involve "signals" mediated through lectin-like molecules on the external surfaces of cells that bind to specific classes of carbohydrate structures on membranes of other cells. Thus, changes in the array of either the lectin-like membrane proteins or the surface glycoconjugates could alter the fate of the cells involved. Eukaryotic lectins have been observed in many systems and there is mounting

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