α -1,4-D-Glucan phosphorylase of gram-positive *Corynebacterium callunae*: isolation, biochemical properties and molecular shape of the enzyme from solution X-ray scattering

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The α -1,4-D-glucan phosphorylase from gram-positive *Corynebacterium callunae* has been isolated and characterized. The enzyme is inducible approx. 2-fold by maltose, but remarkably not repressed by D-glucose. The phosphorylase is a homodimer with a stoichiometric content of the cofactor pyridoxal 5'-phosphate per 88-kDa protein subunit. The specificity constants ($k_{cat}/K_{m,glucan}$) in the directions of glucan synthesis and degradation are used for the classification of the enzyme as the first bacterial starch phosphorylase. A preference for large over small substrates is determined by variations in the apparent binding constants rather than catalytic-centre activities. The contribution

of substrate chain length to binding energy is explained assuming two glucan binding sites in *C. callunae* phosphorylase: an oligosaccharide binding site composed of five subsites and a high-affinity polysaccharide site separated from the active site. A structural model of the molecular shape of the phosphorylase was obtained from small-angle solution X-ray scattering measurements. A flat, slightly elongated, ellipsoidal model with the three axes related to each other as 1:(0.87–0.95):0.43 showed scattering equivalence with the enzyme molecule. The model of *C. callunae* phosphorylase differs from the structurally well-characterized rabbit-muscle phosphorylase in size and axial dimensions.

INTRODUCTION

Pyridoxal 5'-phosphate (PLP)-dependent α -glucan phosphorylases play a central role in the bacterial carbohydrate metabolism pertaining to the mobilization of intracellular storage polysaccharide. In addition, they are responsible for the utilization of malto-oligosaccharides, imported from the extracellular environment to the cytoplasm, like, for example, in gram-negative *Escherichia coli* or *Klebsiella pneumoniae* [1]. The phosphorylase-catalysed reaction represents the first step in the degradation of poly- or oligo-saccharide substrates and results in the release of α -D-glucose-1-phosphate (Glc-1-P):

In eqn. (1), n is the number of glucosyl units which, for maltodextrin substrates, is typically 5–10. *E. coli* has been extensively studied with regard to the individual protein and enzyme components participating in the utilization of maltodextrins (for review, see [1]). Oligosaccharides with a degree of polymerization (DP) of up to 7 can be translocated by transport machinery from the growth medium into the cytoplasm [1–3]. There, maltodextrin phosphorylase and amylomaltase are responsible for the breakdown of the maltodextrins to Glc-1-P and D-glucose. Amylomaltase converts maltotriose and longer oligosaccharides to D-glucose and maltodextrins [4]. Maltodextrin phosphorylase, in turn, liberates Glc-1-P from the non-reducing end of maltodextrins with a $DP \geq 4$ [5,6]. Both enzymes are encoded by the *mal* PQ operon located in the *mal* A region of the *E. coli* maltose regulon [1]. Maltodextrin phosphorylase and amylomaltase are thus co-induced, and induction is seen during growth of the organism on maltose or maltodextrins. In contrast,

maltotriose was shown to function as an exclusive inducer *in vitro* [7]. As implied by its name, the maltodextrin phosphorylase from *E. coli* prefers short oligosaccharides as substrate to polysaccharides such as starch or glycogen [6]. The activity of the phosphorylase is regulated by transcriptional control of synthesis only, and is thus not subject to allosteric or covalent control mechanisms [8,9]. In addition to rabbit-muscle glycogen phosphorylase, the enzyme from *E. coli* has served as a model to study the mechanism of substrate binding and catalysis in α -glucan phosphorylases [5,10–12]. Apart from the absolute requirement for PLP some common features of glucan phosphorylase from various sources are the dimer structure, the molecular mass of the protomer (≈ 90 kDa) and, probably, the kinetic mechanism. Distinguishing features include the regulation of activity, the pattern of inhibition by various metabolites and especially the specificity for the glucan substrate [10,13,14]. The substrate preference is used for a classification of the enzymes in glycogen, starch or maltodextrin phosphorylases.

Little is known about the composition, function and specificity of protein systems that are responsible for utilization of storage glucan, as well as maltodextrin, in gram-positive bacteria. Specifically, the isolation and characterization of glucan phosphorylase or amylomaltase has not, to the best of our knowledge, been studied in representatives of these organisms. We have selected the gram-positive bacterium *Corynebacterium callunae* for two reasons. Firstly, the organism was shown to synthesize α -glucan phosphorylase during growth on maltose or maltodextrins [15]. Secondly, the genus *Corynebacterium* is capable of accumulating an intracellular starch-like polysaccharide [16,17]. The latter aspect seemed interesting because starch is thus a likely candidate representing a physiological substrate of phosphorylase in *C. callunae*, whereas traditionally, bacterial phosphorylases

Abbreviations used: P-mode, glucan phosphorolysis; S-mode, glucan synthesis; DP, degree of polymerization; Glc-1-P, α -D-glucose-1-phosphate; PLP, pyridoxal 5'-phosphate; SAXS, small-angle X-ray scattering; HIC, hydrophobic interaction chromatography.

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are thought to use maltodextrin rather than polysaccharide as substrate [6,13].

In this work, we have studied the formation of α -glucan phosphorylase and amylomaltase in *C. callunae*. Both enzymes are not repressed by D-glucose. The phosphorylase, but strikingly not the amylomaltase, seems inducible by maltose or maltodextrin. The phosphorylase has been isolated, characterized and used for a comparison with its counterpart from the gram-negative bacterium *E. coli*. Most remarkably, the enzyme from *C. callunae* is the first bacterial starch phosphorylase that prefers polysaccharides to small maltodextrins. A model of the molecular shape of a bacterial phosphorylase in solution was derived from solution small-angle X-ray scattering (SAXS) measurements and compared with structural data of rabbit-muscle glycogen phosphorylase.

EXPERIMENTAL

Materials

Glc-1-P, D-fructose-6-phosphate, PLP, pyridoxamine phosphate, nicotinamide coenzymes, ATP, AMP, glycogen, D-glucose-6-phosphate dehydrogenase and non-specific protein kinase from rabbit muscle were obtained from Sigma (Deisenhofen, Germany). Malto-oligosaccharides with a DP of 3–7 and phosphoglucomutase were from Boehringer (Mannheim, Germany). Maltodextrin with an average DP of 9 (dextrin 9) and 11 (dextrin 11) were obtained from Agrana (Gmünd, Austria) and soluble starch was from Merck (no. 152.0250). D-Glucose dehydrogenase (100 units/ml) was from Amano (Milton Keynes, Bucks., U.K.). Phenyl-Sepharose 4B-CL fast flow high sub, Sephadex G 25 coarse, Superose 12 HR 10/30, Mono-P HR 5/20 and Sepharose 6B, carrying an activated bis-2,3-epoxypropoxybutane spacer, were from Pharmacia (Uppsala, Sweden). Cartridges (Econo Pac, 5 ml) prefilled with methyl substituted material for hydrophobic interaction chromatography (HIC) were from Bio-Rad (Richmond, CA, U.S.A.). The strain *C. callunae* DSM 20147 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *E. coli* JM 109, carrying the plasmid pMAP 101 for the overexpression of *E. coli* maltodextrin phosphorylase [18], was kindly given by Dr. R. Schinzel (University of Würzburg, Germany).

Cultivation of the organism and induction of phosphorylase

C. callunae DSM 20147 was cultivated in a 10-litre MBR bioreactor system at 30 °C, 40% air saturation and a constant pH of 7.4 using the basal medium recently described [15]. The medium was supplemented with additional carbon sources such as D-glucose, maltose and dextrin 9 as indicated. At certain times, 250-ml samples were taken from the fermentation mixture, and supernatant and cells were separated by centrifugation (Hermle ZK 401, rotor 6.9, 6000 rev./min, 4 °C, 15 min). The sugar composition of the supernatant was analysed by TLC as follows. Each sample was diluted (1:100), and then 1.5 μ l of this mixture was applied to silica plates (Kieselgel 60; Merck). The plates had been pre-activated by 10-min incubation at 120 °C. The solvent was butanol/propanol/ethanol/water (2:3:3:2, by vol.), and detection was accomplished with the thymol reagent. The centrifuged cell material was diluted (1:3, by wt.), and approx. 3 ml of this mixture was disrupted by repeated passage through a 9.5 mm ($\frac{3}{8}$ in) French pressure cell (Aminco, Silver Springs, MA, U.S.A.) at 138 MPa (20000 lbf/in²). Following

ultracentrifugation (Beckman L-70, rotor Ti70, 30000 rev./min, 4 °C, 20 min) the raw cell extracts were used for further analyses. After 30–35 h cultivation time, when a maximum volumetric phosphorylase activity was obtained, approx. 70–90 g of wet cell material/litre of medium was recovered by centrifugation (see above). The cells were diluted 1:4 (by wt.) in 50 mM potassium phosphate buffer, pH 6.9, containing 20%-satd. (NH₄)₂SO₄, 7 mM β -mercaptoethanol and 10 mM EDTA. Then, cell disruption was carried out at 4 °C in a continuously operated Dyno Mill using glass beads of 0.2 mm diameter and an average disruption time of 10 min. Cell debris and precipitated protein were removed by ultracentrifugation (40000 g, 40 min), and the clear supernatant was used for enzyme isolation. *E. coli* maltodextrin phosphorylase was obtained from shaken flask cultures of the organism induced with 0.2 g/l maltose as recently described in more detail [19].

Enzyme purification

Protein purification was carried out on the Pharmacia FPLC and Gradi Frac systems. The initial purification of *C. callunae* phosphorylase used HIC on a 300-ml Phenyl-Sepharose 4B-CL fast-flow high-sub column [15]. Briefly, approx. 20 mg of protein was applied/ml of gel, and elution was carried out at a flow rate of 400–500 cm/h with a gradient of 20%- to 0%-satd. (NH₄)₂SO₄ in 50 mM phosphate buffer, pH 7.0. The phosphorylase preparation was then concentrated to a volume of less than 50 ml (Amicon ultrafiltration cell; 30-kDa cut-off membrane YM 30) and subjected to gel filtration on a 300-ml column of Sephadex G 25 coarse, equilibrated in 0.1 M triethanolamine buffer (pH 7.6)/10 mM EDTA. The resulting enzyme preparation was kept on ice until loaded onto an affinity column of maltodextrin-Sepharose 6B equilibrated in the same buffer. The coupling of dextrin 9 to the pre-activated resin was carried out according to the supplier's instructions (Pharmacia). An 8-ml affinity column (XK 26, Pharmacia) was employed that had a binding capacity of approx. 15–20 mg of phosphorylase protein (equivalent to 450–600 enzyme units). Elution was accomplished at 2.5 ml/min using a step gradient of 0.0, 0.1 and 0.5 M NaCl. Fractions of 1.2 ml were collected, and those containing phosphorylase activity were pooled. Then, after potassium phosphate (1 M) and (NH₄)₂SO₄ (3.2 M) had been added to final concentrations of 50 mM and 25%-satd. respectively, the resulting protein preparation was applied to a 5-ml Econo-Pac Methyl-HIC column. Elution was carried out at 6 ml/min with a step gradient using decreasing concentrations of (NH₄)₂SO₄ (25%-, 12%-, 0%-satd.). The maltodextrin phosphorylase from *E. coli* was purified to apparent electrophoretic homogeneity by the two-step procedure described recently [19].

Electrophoreses

Each purification step was controlled by SDS/PAGE and non-denaturing anionic PAGE (using PhastGel Gradient 8–25 in both cases), carried out on a Pharmacia PhastSystem unit. Staining for phosphorylase activity was performed by assaying in the direction of phosphorolysis following non-denaturing PAGE. The gel was incubated at pH 6.9 in the presence of the components of the assay mixture containing 30 g/l maltodextrin and 0.8 g/l NAD⁺ (see the Assays section), as well as 5-methylphenazine methosulphate (2 mg/ml) and Nitroblue Tetrazolium chloride (6 mg/ml) as electron acceptors. The control did not contain maltodextrin. Visualization of protein bands was by Coomassie Brilliant Blue or silver staining, or both.

Quarternary structure, molecular mass, isoelectric point

The molecular mass of phosphorylase subunits was determined by SDS/PAGE (see Electrophoreses section above) and that of the native enzyme was determined by gel filtration on Superose 12 HR 10/30. The column was equilibrated in 67 mM phosphate buffer/0.1 M NaCl, pH 7.0, and protein elution was carried out at a constant flow rate of 0.5 ml/min. For the calibration cytochrome *c* (12.4 kDa), myoglobin (17.6 kDa), β -lactoglobulin (35 kDa), egg albumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), *E. coli* phosphorylase (180 kDa), horse ferritin (480 kDa) and thyroglobin (669 kDa) were used as standards.

Chromatofocusing was carried out on a Mono-P HR 5/20 column. An appropriate amount of enzyme was applied to the column that was equilibrated with 20 mM phosphate buffer, pH 7.3. Elution of the phosphorylase was carried out using Polybuffer 74, pH 4.0 (Pharmacia). Fractions of 1 ml were collected, and the pH of the fraction that contained the enzyme was measured. Isoelectric focusing and electrophoretic titration curve analysis were done using precast Phastgel IEF 3-9. An isoelectric calibration kit (Pharmacia) was used as standard and silver staining was employed for visualizing protein bands.

N-terminal sequencing and amino acid analysis

The N-terminal sequencing was carried out from 300 pmol of phosphorylase in 50 μ l of phosphate buffer. The sample was applied to a glass-fibre disc, dried under N_2 and then subjected to automatic pulsed-liquid Edman degradation in a Model 477 A gas-phase protein sequencer (Applied Biosystems). On-line analysis of the phenylthiohydantoin derivatives was performed under standard operating conditions of the sequencer, and the first ten amino acids were determined. Comparison of the N-terminal sequences of various phosphorylases was made using the program Clustal [20]. For total amino acid analysis, phosphorylase (1.0 mg/ml) was dried under N_2 and subjected to hydrolysis *in vacuo* using 6 M HCl. The hydrolysis was carried out at 110 °C for 24 h and 48 h. The dried hydrolysate was dissolved in 0.2 M potassium borate buffer, pH 9.5, and then subjected to analysis by reversed-phase HPLC using pre-column derivatization of the amino acids with *o*-phthalaldehyde [21]. Fluorescence detection (excitation 340 nm/emission 450 nm) was employed, and norleucin was used as internal standard. A search for phosphorylases with similar total amino acid composition deposited in the Swissprot and PIR protein databases was done employing the programme PropSearch [22]. The molecular mass of the new phosphorylase but not its pI was included and given a weight factor of 10.

Assays

Phosphorylase and all other enzyme activities were routinely assayed at 30 °C using 50 mM Tris/HCl buffer, pH 6.9. If not indicated otherwise, dextrin 9 was used as substrate for phosphorylase. In the direction of phosphorolysis (glucan phosphorolysis, P-mode) the continuous, coupled photometric test was applied as previously described [15]. The concentration of phosphate was 50 mM, and that of dextrin 9 was 30 g/l and 10 g/l with the phosphorylases from *C. callunae* and *E. coli* respectively. All phosphorylase units are expressed as P-mode activity and refer to 1 μ mol of NADH formed/min. P-mode phosphorylase activity at temperatures above 30 °C, in the presence of inhibitors, effectors or phosphate concentrations higher than 50 mM, was measured discontinuously. Hence, samples taken at different time points were heat-treated, and then the concentration of Glc-1-P was determined by diluting aliquots into the

coupled enzyme assay [15]. In the direction of glucan synthesis (S-mode) a discontinuous measurement was used, and the concentration of P_i released from Glc-1-P was determined by the Spectroquant phosphate kit (Merck) or by the assay described by Drueckes et al. [23]. All measurements were corrected for the appropriate blank readings. Arsenolysis of maltohexaose (24 mM) by phosphorylase was measured in the presence of up to 150 mM arsenate, and the release of D-glucose was determined using the hexokinase-based assay. Amylomaltase was quantified in a coupled, continuous or discontinuous assay with D-glucose dehydrogenase [4]. The substrate concentration was 10 mM maltotriose. Glucan synthase, glucose-6-phosphate dehydrogenase, phosphoglucomutase, alkaline phosphatase and NADH oxidase were measured according to Bergmeyer [24]. Protein was determined by the method of Bradford [25] using BSA as standard.

Kinetics

The kinetic constants were determined at 30 °C in the P- and S-mode of the reaction. One substrate was varied over a concentration range of approx. 0.5–10 times K_m , the respective Michaelis constant. The other substrate was kept constant at a saturating concentration. The substrate degradation was monitored over a period of approx. 1 h with aliquots taken from the reaction mixture at intervals of 10 min. With substrate depletion being usually less than 5%, the reaction time courses were linear and used directly for the calculation of reaction rates. All kinetic constants were calculated by non-linear regression methods, fitting the observed rate data to a rectangular hyperbola. An extension of the hyperbola accounting for substrate inhibition was included as required (eqn. 2).

$$V(S) = k_{cat} \cdot E \cdot S / (K_{m,S} + S + S^2/K_{is}) \quad (2)$$

In eqn. (2), $V(S)$ represents the reaction rate, k_{cat} the catalytic constant, E the total concentration of phosphorylase monomers, S the varied substrate, $K_{m,S}$ the apparent Michaelis constant for S and K_{is} the substrate inhibition constant for S .

Spectroscopic measurements

PLP was quantified according to Wada and Snell [26]. All absorption and emission spectra were recorded on a Hitachi spectrofluorimeter F 2000 at 25 ± 1 °C in cells of 1 cm optical path. A scanning speed of 1 nm/s and slit widths of 5 nm for the excitation and emission wavelengths were used. UV spectra were recorded on a Shimadzu UV160 spectrometer.

NMR

^{31}P -NMR measurements were performed at 121.49 MHz on a Bruker AM300SWB superconducting spectrometer, as previously described [27]. The phosphorylase from *C. callunae* was dialysed against 50 mM Mes/1 mM EDTA/1 mM dithioerythritol/50 mM KCl, pH 7.0, and protein concentrations of 0.1–0.3 mM were employed. All chemical shifts are reported relative to 85% (v/v) H_3PO_4 .

SAXS

Protein stock solutions of 15 mg/ml pure phosphorylases from *C. callunae* and *E. coli* in 0.1 M potassium phosphate buffer, pH 7.0, were prepared by repeated concentration in ultrafiltration tubes (Centriprep 30 kDa cut-off). Scattering experiments were performed using a Kratky compact camera with a slit collimation system and a proportional counter tube. The Philips PW 2253/11

X-ray generator, with a fixed tube copper target, was operated at 50 kV and 30 mA. To eliminate polychromatic effects, a pulse-height discriminator (adjusted to the CuK_α line) was employed in combination with a numerical elimination of the CuK_β contribution. SAXS measurements were performed at $4 \pm 0.1^\circ\text{C}$ in Mark capillary tubes of 1 mm diameter with four different concentrations of the protein sample ranging from 5 to 15 mg/ml. The background scattering of buffer was recorded separately. Scattering intensities were measured at 114 different angles from 0.0019 to 0.105 rad, corresponding to values of the scattering vector, h , of $0.0776\text{--}4.268\text{ nm}^{-1}$. The vector h is given by $(4\pi/\lambda) \sin\Theta$ (2Θ is the scattering angle in radians and λ is the wavelength of the X-ray, i.e. 0.154 nm for the CuK_α line). Each scattering curve was recorded several times to reduce errors. After the necessary corrections for background scattering the numerical monochromatization was performed simultaneously with a desmearing procedure (corrections for beam geometry) using the programme ITP [28,29]. This strategy of data evaluation leads to desmeared scattering curves, $I(h)$ and distance distribution functions, $p(r)$, where $I(h)$ is the scattered intensity in pulses per second as a function of the scattering vector and $p(r)$ is the distance distribution as a function of distances r within the particle. The scattering curve at infinite dilution was obtained from the extrapolation of a series of scattering data at different protein concentrations. The radius of gyration, R_{gyr} , was calculated according to eqns. (3a) and (3b).

$$R_{\text{gyr}}^2 = -3 \frac{d \ln I(h)}{d(h^2)} \quad (3a)$$

$$R_{\text{gyr}}^2 = \frac{\int_0^\infty p(r) r^2 dr}{2 \int_0^\infty p(r) dr} \quad (3b)$$

The hydrated volume of the protein particle was calculated according to eqn. (4).

$$V = 2\pi^2 \frac{I_0}{Q} \quad (4)$$

where I_0 is the scattering intensity at zero angle and

$$Q = \int_0^\infty I(h) h^2 dh$$

Other details of the evaluation procedure are given elsewhere [29].

Model calculations

A method of finite elements was used to calculate structural models that would fit the experimental $I(h)$ and $p(r)$ data. The models were approximated by a finite number of small spheres of equal size. The diameter of these spheres was chosen in such a way that the volume of the model was represented exactly by the sum of the volumes of all spheres (which corresponded to a slight formal overlap between adjacent spheres). Models were generated in a stepwise manner to improve the agreement between the experimental and theoretically calculated scattering data. Model discrimination was based on the criteria that the model should yield the same radius of gyration, the same maximum particle dimensions and represent a good fit of the experimental scattering curve and the distance distribution function.

RESULTS

Formation of phosphorylase in *C. callunae*

The glucan phosphorylase activities in cell-free extracts of *C. callunae* grown on maltose, maltodextrin or D-glucose were

Figure 1 Formation of α -1,4-D-glucan phosphorylase in *C. callunae*

The time-dependent synthesis of glucan phosphorylase ($\square$), amyloamylase ($\blacktriangle$) and glucan synthase ($\blacksquare$) related to sugar consumption was monitored. The organism was grown at 30°C on basal medium supplemented with 60 g/l D-glucose and 20 g/l maltose. The enzyme activities in raw cell extracts of samples taken during the cultivation were measured, and values are based on the total amount of protein in the extracts (U/mg protein = units/mg of protein). The sugar consumption was monitored by subjecting diluted aliquots of the culture liquid to TLC. For further details, see the Experimental section. The arrow indicates the time-point of D-glucose depletion.

compared. The control experiment contained no added sugar and yielded a total enzyme activity of 5.1 units/g biomass and a specific enzyme activity 0.12 units/mg of protein. These activities were 2- to 2.5-fold increased in cells induced by maltose (5 g/l) or maltodextrin (12 g/l). No repression of the enzyme by D-glucose in concentrations from 5 to 30 g/l seemed to occur. The sugar had no appreciable effect on the specific phosphorylase activity relative to the control. To obtain sufficient quantities of phosphorylase for enzyme isolation, we tested whether high yields of biomass (obtained on D-glucose) and efficient induction by maltose could be achieved in one cultivation experiment. Of a mixture of D-glucose and maltose, *C. callunae* preferentially used D-glucose (Figure 1). Maltose consumption started when the D-glucose was close to depletion. Concomitantly, an increase in specific phosphorylase activity was observed that approached the value observed when maltose was used alone. Induction by maltose did not result in a simultaneous increase in either glucan phosphorylase and amyloamylase activities in *C. callunae* (Figure 1). The time course of formation of amyloamylase in Figure 1 seemed to parallel that of glucan synthase rather than that of glucan phosphorylase. Maltose could be replaced with maltodextrin as an inducing component. However, the limit size of the oligosaccharide is 4 glucosyl units: dextrans with DP 4 are neither imported nor extracellularly degraded by *C. callunae*.

Purification of phosphorylase from *C. callunae*

The α -glucan phosphorylase from *C. callunae* was purified to apparent homogeneity by a 4-step procedure that is summarized in Table 1. The isolated enzyme had a specific activity of 33 ± 3.0 units/mg and was recovered in an overall yield of 55%. An efficient first step in the purification of the enzyme is HIC on Phenyl-Sepharose [15]. Then, affinity chromatography on maltodextrin-Sepharose 6B was used to remove the majority of other protein contaminants (Figure 2, top). Surprisingly, the phosphorylase did not bind to glycogen-glycine-Sepharose 4B-CL, which is used to isolate the *E. coli* maltodextrin phosphorylase. The *C. callunae* phosphorylase, obtained in yields of $> 60\%$, had a specific activity of 27–30 units/mg and, as judged by SDS

Table 1 Purification of the α -1,4-D-glucan phosphorylase from *C. callunae*

The results were obtained from processing 116 g of wet biomass of *C. callunae*. For other details see the Experimental section. '+' denotes detectable amylomaltase by non-denaturing anionic PAGE and by measurement of enzyme activity; '-' not detectable.

	Total activity (units)	Total volume (ml)	Specific activity (units/mg)	Yield (%)	Purification factor (-fold)	Amylomaltase
Cell extract	1081	865	0.20	100	—	+
Hydrophobic chromatography (phenyl ligand)	1001	275	4.30	93	21	+
Ultrafiltration/gel filtration	858	116	3.44	80	17	+
Affinity chromatography	673	89	26.70	62	132	+
Hydrophobic chromatography (methyl ligand)	594	60	33.53 $\pm$ 3.00	55	165	—

Figure 2 Purification of phosphorylase from *C. callunae*

The peaks containing phosphorylase activity are shaded. The composition of the elution buffer is indicated as a percentage of buffer B. See the Experimental section for details. Top: elution profile on maltodextrin-Sepharose 6B. Buffer A contained 0.1 M triethanolamine/10 mM EDTA, pH 7.6. Buffer B was as buffer A with 500 mM NaCl. Bottom: elution profile on Econo-Pac-Methyl-HIC. Buffer A contained 50 mM potassium phosphate, pH 6.9, with 25%-satd. $(\text{NH}_4)_2\text{SO}_4$, whereas buffer B contained no $(\text{NH}_4)_2\text{SO}_4$.

PAGE, was greater than 98% pure. However, when this preparation was incubated at 30 °C with maltotriose (50 mM) in the absence of phosphate, and aliquots were withdrawn from the reaction mixture at regular intervals up to 1 h and subjected to analysis by TLC, a significant, time-dependent synthesis of larger oligosaccharides and D-glucose was observed. This activity could not be due to phosphorylase, and the product pattern matched up exactly to the proposed action of amylomaltase with oligosaccharides, reported with the enzyme from *E. coli* [4]. In the presence of P_i , contamination by amylomaltase was found to

Figure 3 Enzyme purification documented by SDS/PAGE

Lanes 1–6 left to right. Lane 1, molecular mass standards; lane 2, pure phosphorylase; lane 3, fraction after affinity chromatography; lane 4, fraction after Phenyl-Sepharose chromatography; lane 5, raw cell extract. The standards were, bottom to top, lactalbumin (14.1 kDa), carboanhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase *b* (94 kDa). All samples were reduced and then alkylated by iodoacetamide. Staining with Coomassie Brilliant Blue and silver was used for visualizing protein bands.

fake P-mode phosphorylase activity with maltotriose or maltotetraose as substrate. Amylomaltase-free phosphorylase was then obtained by chromatography on an Econo-Pac Methyl-HIC column, and a typical elution profile is shown in Figure 2, bottom.

The purified enzyme migrated as a single protein band in SDS/PAGE (Figure 3) and as a broad band in non-denaturing anionic PAGE, that was identified as phosphorylase by staining for enzyme activity (results not shown). One single protein band or migration curve was observed in isoelectric focusing and electrophoretic pH titration analysis respectively.

Physicochemical properties

The results of SDS/PAGE demonstrated that *C. callunae* phosphorylase is composed of one or more identical subunits with a molecular mass of 88 kDa. Gel-filtration chromatography revealed a molecular mass of the native protein of 176 kDa. Hence, the enzyme is a dimer of two identical protomers. The protein did not migrate below pH 6.8 in isoelectric focusing and pH titration analysis. It was not eluted from a MonoP chromatofocusing column in the pH range 4–7 unless 1 M NaCl was added to the elution buffer. Its exact pI thus remained uncertain but was ≤ 6.8 .

Table 2 Total amino acid composition of *C. callunae* glucan phosphorylase

Results are means of two independent determinations including hydrolysis. For methodology see the Experimental section. Cys was measured by titration with Ellman's reagent of reactive thiols in phosphorylase denatured in SDS (4%, by wt.); Trp and Pro were not determined.

Amino acid	Mol of amino acid/mol of subunit of 88 kDa	Amino acid	Mol of amino acid/mol of subunit of 88 kDa
Arg	43.4	Val	30.8
Glx	73.4	Met	15.4
Ser	29.4	Ile	29.8
His	14.6	Leu	56.6
Gly	38.9	Phe	23.6
Thr	37.0	Lys	20.4
Ala	67.0	Cys	5.3
Tyr	25.3	Asx	79.8

N-terminal sequence and total amino acid composition

The N-terminal decapeptide of the phosphorylase is Pro-Glu-Lys-Gln-Pro-Leu-Pro-Ala-Ala-Leu. It is entirely different from N-terminal regions of other glucan phosphorylases. The total amino acid composition of the protein is shown in Table 2. Using the program PropSearch [22], the amino acid composition of the phosphorylases from *E. coli*, *Solanum tuberosum* and rabbit muscle have been found to be similar to that of the enzyme from *C. callunae*. The significance was in the range 68–75% (cf. [22] for the evaluation principle).

Cofactor analysis

Using the protocol of Shaltiel et al. [31,32] for the preparation of the apoenzyme of glycogen phosphorylase, *C. callunae* phosphorylase was inactivated to less than 1% residual activity. Partial reactivation of the phosphorylase was obtained following a 60-min incubation of the gel-filtered enzyme in the presence of PLP [25 µM, Tris buffer (pH 6.9)/50 mM P_i]. In contrast, pyridoxamine phosphate was ineffective. These results suggest that the phosphorylase from *C. callunae* contains PLP as cofactor whose carbonyl group is essential for binding. The cofactor content is 0.87 ± 0.10 mol/subunit of protein. The absorption spectrum of the enzyme showed a weak absorption band at 330 nm likely to reflect the absorption of the cofactor. A comparison of the fluorescence emission spectra in phosphorylases from *C. callunae* and in *E. coli* is shown in Figure 4. When excited at 330 nm, both enzymes exhibited emission spectra between 470 and 550 nm that are characterized by their large Stokes shift. The enzymes were, however, clearly different in the emission maximum and the quantum yield of PLP fluorescence. Maximum fluorescence was seen at 512 nm and 532 nm with phosphorylase from *C. callunae* and *E. coli* respectively. Unlike maltodextrin phosphorylase from *E. coli*, the enzyme from *C. callunae* is prone to chemical quenching of the cofactor fluorescence by iodide in concentrations of 0.05–0.25 M [50 mM Tris buffer (pH 6.9)/10 mM P_i]. Thus PLP in *C. callunae* phosphorylase seemed to be relatively more exposed to the aqueous solvent. Following excitation of intrinsic protein fluorescence at 280 nm, energy-transfer spectra were recorded between 450 and 550 nm. Fluorescence emission was detectable only with the enzyme from *E. coli* (Figure 4, inset C). A ³¹P-NMR experiment carried out at pH 7.04 revealed a chemical shift of 3.85 p.p.m. downfield from the phosphoric acid reference for the 5'-phosphate group of PLP in *C. callunae* phosphorylase (results not

Figure 4 Comparison of the fluorescence emission spectra of glucan phosphorylase from *C. callunae* and maltodextrin phosphorylase from *E. coli*

The excitation wavelength was 330 nm, and the protein concentration was 0.05 mg/ml in 50 mM Tris buffer, pH 6.9, supplemented with 20 mM P_i (25 °C). A, *C. callunae* phosphorylase; B, *E. coli* maltodextrin phosphorylase. The inset with curve C shows the fluorescence of *E. coli* phosphorylase following excitation at 280 nm.

shown). This shift is similar to that reported for the *E. coli* maltodextrin phosphorylase (3.60 p.p.m.; [27]).

Enzyme activity dependence on pH and temperature

The pH optima in P- and S-mode are 7.0–7.2 and 5.7–6.1 respectively. The pH-activity profiles were almost symmetrical in either direction of reaction. The apparent pK_a and pK_b values in glucan synthesis were estimated to be pH 5.3 and pH 6.6, and that in phosphorolysis to be pH < 5.0 and pH 8.25. The pK_a is likely to reflect instability of the enzyme because the phosphorylase was rapidly denatured below pH 4.5. The temperature optimum of the enzyme in P-mode direction was 45 °C. The enzyme activity increased linearly from 15 to 42 °C resulting in a 5-fold activation of the phosphorylase.

Effectors and inhibitors

The compounds AMP (1–20 mM), Na₂SO₄ or (NH₄)₂SO₄ (50–300 mM each) had a weak inhibiting rather than activating effect on the bacterial phosphorylase described here. Preincubation of the enzyme with non-specific mammalian protein kinase (or a crude cell extract from *C. callunae*) and ATP plus Mg²⁺ (1 mM) for 60 min, affected neither the catalytic constant nor the substrate binding constants of the phosphorylase. Inhibition in P-mode direction was seen with various typical inhibitors of glucan phosphorylase: D-glucose (50 mM), D-glucono-δ-lactone (0.05 mM), ATP (5 mM) and ADP (5 mM) caused 30%, 90%, 10% and 8% inhibition respectively. Caffeine and glucose 6-phosphate (both at 5 mM) are poor inhibitors, and no synergism of the carbohydrate- and purine-type inhibitors was detected. α- and β-cyclodextrin (5 and 10 mM) had no effect on the activity of the *Corynebacterium* enzyme. Vanadate and molybdate (5 mM) inhibited phosphorylase activity by more than 50%.

Specificity and kinetics

The results of the kinetic characterization of *C. callunae* phosphorylase, including the aspect of specificity in P-mode and S-

Table 3 Apparent kinetic constants of *C. callunae* phosphorylase in the direction of glucan (a) degradation and (b) synthesis

The reaction conditions were (a) 30 °C and pH 6.9, (b) 50 mM Tris buffer, 30 °C and pH 6.0, and the calculation of the catalytic constant k_{cat} was based on a molecular mass of 88 kDa of the phosphorylase monomer. The K_m values represent the apparent Michaelis constants determined at saturation with the second substrate. For other details, see the Experimental section. DP, average length of linear chain.

Variable substrate	Fixed substrate	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)
(a) Degradation				
Maltopentaose	50 mM P _i *	33.8 ± 1.8	2.73 ± 0.54	12.4
Maltohexaose	50 mM P _i	38.9 ± 0.9	3.15 ± 0.23	12.3
Maltoheptaose	50 mM P _i	41.3 ± 1.1	3.72 ± 0.23	11.1
Dextrin DP 9	50 mM P _i	47.3 ± 1.4	1.22 ± 0.15	38.8
Dextrin DP 11	50 mM P _i	50.0 ± 3.8	0.50 ± 0.03	100.0
Glycogen DP 14	50 mM P _i	34.5 ± 2.1	0.27 ± 0.02	127.8
Starch DP 24	50 mM P _i	69.9 ± 2.2	0.04 ± 0.01	1747.5
Dextrin DP 11	300 mM P _i †,‡	51.7 ± 3.8	6.22 ± 0.78	8.3
P _i	18 mM Dextrin DP 9*	50.5 ± 2.2	1.18 ± 0.22	42.7
P _i	18 mM Dextrin DP 9§	49.7 ± 2.0	20.51 ± 2.34	2.4
Arsenate	24 mM Maltohexaose*	4.90 ± 0.12	22.70 ± 1.69	0.2
(b) Synthesis				
Maltoheptaose	30 mM Glc-1-P	312.4 ± 21.2	39.86 ± 6.02	7.8
Dextrin DP 11	30 mM Glc-1-P	187.4 ± 14.5	46.48 ± 5.96	4.0
Glycogen DP 14	30 mM Glc-1-P	283.4 ± 14.2	4.32 ± 0.86	65.5
Starch DP 24*	30 mM Glc-1-P	300.0 ± 20.4	0.47 ± 0.11	638.3
Glc-1-P	50 mM Dextrin DP 11*	131.0 ± 25.2‡	1.04 ± 0.15	126.0

|| Molar concentrations of polysaccharides are calculated from the average chain length (DP) with available non-reducing ends. The values for potato starch and glycogen are from [42] and [43] respectively.

* 50 mM Tris/HCl buffer, pH 6.9.

† 300 mM potassium phosphate buffer.

‡ Discontinuous assay.

§ Range of 0–1.7 M P_i.

Figure 5 Phosphorolysis of maltoheptaose by phosphorylase from *C. callunae*

Maltoheptaose (10 mM) was incubated at 30 °C in 0.25 M phosphate buffer, pH 6.9, with 1 unit/ml phosphorylase. Aliquots taken at the times indicated below were diluted (1 mg of sugar/ml), and 1.5 µl of the diluted sample analysed by TLC on pre-activated silica plates (see the Experimental section for details). Lanes 1–9 left to right. Lane 1, Glc-1-P; lane 2 (bottom to top), maltoheptaose, Glc-1-P and maltohexaose to glucose; lanes 3–9, samples taken after 3, 20, 40, 60, 80, 120 and 240 min reaction time.

modes, are summarized in Table 3. Pertaining to α -1,4-D-glucan substrates differing in average molecular mass and degree of substitution, with α -1,6-glycosidic linkages, a broad specificity of the enzyme was found. In contrast, only arsenate could replace phosphate in the glucan degradation direction, albeit with at least 10-fold lower catalytic efficiency (Table 3a). The phos-

phorylase was inactive with phosphite, fluorophosphate, thio-phosphate and PP_i (50 mM each). Glc-1-P could not be replaced either by β -D-glucose-1-phosphate or by α -D-galactose-1-phosphate, D-glucuronic acid-1-phosphate or D-galacturonic-1-phosphate. The catalytic centre activities in P-mode were 4- to 7-fold lower than in S-mode (Tables 3a and 3b). The apparent binding constants for various α -glucans point to starch as the preferred substrate of *C. callunae* phosphorylase. The specificity constants in P-mode or S-mode were up to two orders of magnitude higher with polysaccharides than small oligosaccharides. The effect was mainly due to better apparent binding of the large substrates. However, the variation in catalytic constants was not as pronounced. When calculations of binding constants were based on mass concentration rather than molarity of the glucan, the results differed in quantitative terms, but left the conclusions unaffected. Maltotetraose functioned as the shortest primer molecule elongated in S-mode, and maltopentaose was the smallest substrate degraded in P-mode with significant activity. Interestingly, glucose residues 6 and 7 in maltohexaose and maltoheptaose respectively had no effect on the kinetic constants relative to maltopentaose (Table 3a). According to (k_{cat}/K_m) values in Tables 3a and 3b, phosphorolysis is favoured over synthesis with all types of glucan substrate tested. The effect is due to the approx. 10-fold higher apparent K_m values for the glucan in S-mode than in P-mode. It is noteworthy that substrate inhibition was observed with polysaccharides as well as maltodextrins in the direction of glucan phosphorolysis but not in that of glucan synthesis. The apparent binding constant for the inhibitory binding mode of maltodextrin, K_{is} , is > 400 mM. The inhibition was weak compared with that seen with *E. coli* maltodextrin phosphorylase ($K_{\text{is}} = 77$ mM). When maltodextrin had been pretreated with pullulanase, cleaving the α -1,6-

Figure 6 SAXS of the phosphorylase molecule from *C. callunae*

Scattering was recorded at four protein concentrations from 5 to 15 g/l. Top: Lin-log plot of the experimental normalized scattering curve ($\square$) and the theoretical scattering curve (solid line) calculated from the ellipsoidal model structure with three axes related to each other as $a/b/c = 1:0.95:0.42$. Bottom: Lin-Lin plot of the experimental distance distribution function ($\square$) and the theoretical $p(r)$ function (solid line) derived from the ellipsoidal model as in Figure 6, top. The error bars in both parts of the Figure represent the mean deviation between results obtained with different concentrations.

glycosidic linkages [15], substrate inhibition of the phosphorylase from *E. coli* was reduced considerably ($K_{is} > 250$ mM). The apparent binding constants of orthophosphate and dextrin, determined in Tris buffer, were 20- and 5-fold lower respectively than those measured in phosphate buffer (Table 3a). The reason is uncertain, but the effect is not due to a variation of K_m with ionic strength. The apparent binding constants of P_i and dextrin 11 in Tris buffer supplemented with 0.1, 0.3 and 0.5 M NaCl were identical within the statistical significance.

Time course of phosphorolysis

Maltoheptaose was depolymerized by phosphorylase from *C. callunae* in a sequential manner. In spite of the incomplete resolution of the oligosaccharide products in TLC shown in Figure 5, it is evident that the formation of both the hexamer and the pentamer preceded the conversion of maltoheptaose to the limit dextrin maltotetraose. The time-dependent product pattern

Figure 7 Model of the solution structure of *C. callunae* phosphorylase

(a) Top view showing the 2-fold symmetry. (b) Side view in y -direction. (c) Side view in x -direction. The model consisted of 4612 spheres, and the radius of each sphere was 0.247 nm.

obtained with *E. coli* maltodextrin phosphorylase was identical with that shown in Figure 5.

Molecular shape from SAXS

Structural analyses were carried out with the enzymes from *C. callunae* and *E. coli*. In concentrations of 10 and 15 mg/ml, the maltodextrin phosphorylase from *E. coli* seemed to exist in equilibrium between the dimeric form and an appreciable fraction of other polymeric forms. The interparticle interferences hampered the interpretation of the scattering curves, especially of the outer parts of the curves at low scattering angles. With the enzyme from *C. callunae* this complication did not occur, and no apparent damage was done to the protein by irradiation. The average desmeared scattering curve, $I(h)$, obtained with this protein is shown in Figure 6, top. The mean deviation represented by error bars in Figure 6, top, demonstrates that the agreement

between scattering curves obtained with different concentrations of phosphorylase was very good. Hence, the concentration dependence of the radius of gyration was negligible. The corresponding electron-pair distance distribution function, $p(r)$, is shown in Figure 6, bottom. The results in Figures 6 were used to derive the following structural parameters of the phosphorylase from *C. callunae*: radius of gyration (R_{gyr}) 3.89 ± 0.02 nm, radius of gyration of the cross-section (R_c) 2.44 ± 0.01 nm, radius of gyration of thickness (R_t) 1.13 ± 0.01 nm, maximal dimension (D_{max}) 11.80 ± 0.01 nm and volume 290 ± 14 nm³. Based on a specific volume of 0.729 ml/g and a hydration of 0.3 g of water/g of protein, the molecular mass was estimated to be 170 kDa, which agreed well with the results obtained by gel filtration (176 kDa).

The absence of distinct submaxima in the experimental scattering curves indicated that the phosphorylase had an anisometric structure. To obtain a model of the molecular shape of the enzyme, an iterative procedure was used starting by assuming a simple ellipsoidal structure of the protein dimer with semiaxes a , b and c . A comparison of the experimental $p(r)$ and $I(h)$ data with the theoretical calculations based on the ellipsoidal model revealed significant discrepancies between both. To take into account distinct structural features of the protein, models were then built on the basis of flat ellipsoids consisting of small spheres. The best model was composed of 4612 spheres of 0.49 nm diameter and yielded theoretical $p(r)$ and $I(h)$ curves that were in very good agreement with the experimental scattering (Figure 6). The visual representation of this structural model is shown in Figures 7(a) to 7(c). The calculated molecular parameters for the model structure were: R_{gyr} 3.88 nm, R_c 2.47 nm, R_t 1.14 nm, D_{max} 11.8 nm and volume 293 nm³. The maximum dimensions of the protein were 12.13 nm, 11.50 nm and 5.21 nm in x -, y - and z -directions respectively, corresponding to an axial ratio of 1:0.95:0.43. Calculations based on various related models showed that the distance distribution function and the scattering curve changed dramatically upon variation of the axial ratios. However, holding the ratio of axes $a/c = 1:0.43 (\pm 0.01)$ constant, a variation of the length of the semiaxis b to an axial ratio a/b of 1:0.87 was possible without significant changes in the corresponding $p(r)$ and $I(h)$ data.

DISCUSSION

The α -glucan phosphorylase from Gram-positive *C. callunae* has been characterized biochemically and structurally at low resolution. In contrast with several bacterial, plant or mammalian phosphorylase systems [13,14], no evidence has been found that multiple forms of glucan phosphorylase exist in *C. callunae*. The enzyme described here differs in several aspects to phosphorylases isolated from other sources.

Like many bacterial glucan phosphorylases, the enzyme from *C. callunae* is inducible by maltose or maltodextrins. However, unlike the situation in Gram-negative *E. coli*, the phosphorylase and the amylomaltase are not co-induced in *C. callunae*. Hence, an identical organization of glucan phosphorylase and amylomaltase into an operon like the maltose operons described for *E. coli* or *K. pneumoniae* does apparently not exist for *C. callunae* [1]. In contrast with its counterpart from *E. coli*, the *C. callunae* phosphorylase is not subject to catabolite repression by D-glucose. Recently, a glycogen phosphorylase from the Gram-negative thermophile *Thermus thermophilus* was also found not to be repressed by D-glucose [30].

The known α -glucan phosphorylases are dimers composed of two identical ≈ 90 -kDa subunits, each containing one molecule of bound PLP [10,13]. So is the phosphorylase from *C.*

callunae. The absorption and fluorescence properties of the cofactor are crucially dependent on the polarity of the micro-environment [33]. Accordingly, PLP in phosphorylase from *C. callunae* is located in a non-polar region of the enzyme. The fluorescence spectrum of Schiff base PLP derivatives is known to be blue-shifted upon transfer from non-polar to aqueous solvents. From a comparison of the corresponding emission spectra, the cofactor environment in the enzyme from *C. callunae* appeared more hydrophilic than in *E. coli* maltodextrin phosphorylase, and, judged from fluorescence quenching by iodide, more accessible to solvent.

The N-terminal decapeptide of the *C. callunae* phosphorylase protomer is totally different from N-termini of phosphorylases from other sources. This result is in agreement with the conception of highly variable N-terminal regions in most glucan phosphorylases where, as opposed to the more strictly conserved catalytic domain, regulatory sites have evolved with time [8,9,34]. In *C. callunae*, regulation of phosphorylase activity seems to occur by inductive control of synthesis only. In contrast with the weak k_{cat} -affecting activation of *E. coli* phosphorylases by AMP [13], the nucleotide did not activate the *Corynebacterium* enzyme. Sulphate ions activate *E. coli* glycogen phosphorylase [13], but, in contrast, weakly inhibited the enzyme from *C. callunae*. A binding site for D-glucose 6-phosphate is thought to be well conserved in the non-regulated phosphorylases, and inhibition by this phosphorylated sugar was proposed to represent an early control mechanism of phosphorylase activity [9]. Remarkably, D-glucose 6-phosphate was no inhibitor of *C. callunae* phosphorylase. Cyclodextrins (DP 6–8) are good inhibitors of plant-starch phosphorylase [14,35]. The activity of the *C. callunae* enzyme seemed totally unaffected by cyclohexa-amylose, as well as cyclohepta-amylose. The lack of strong inhibition by D-glucose, nucleotides such as ATP or UTP, and purines such as caffeine, distinguishes the bacterial phosphorylase described here from other regulated phosphorylases [14,36,37].

Catalytic properties

Several catalytic properties of *C. callunae* phosphorylase seem similar to those of known α -glucan phosphorylases [5,10,13,14]. From kinetic studies in P-mode with oligosaccharides of different lengths, we propose a glucan-binding site in the active site of *C. callunae* phosphorylase that is composed of five consecutive glucose subsites. The marked decrease in catalytic competence between maltopentaose and maltotetraose highlights the role of the fifth subsite for the phosphorylase reaction. Judged from the corresponding (k_{cat}/K_m) values with maltohexaose and maltoheptaose, the contribution to binding energy of additional subsites 6 and 7 was not significant. D-Glucono-1,5-lactone, a transition-state analogue of the phosphorylase reaction [38], is an effective inhibitor of *C. callunae* phosphorylase in micromolar concentrations. Vanadate and molybdate, structural analogues of phosphate [39,40], are good inhibitors of the *C. callunae* enzyme. Maltoheptaose is degraded by a sequential mechanism that could imply association and disassociation of enzyme and glucan substrate as an integral part of the catalytic cycle.

Substrate specificity

The interaction of phosphorylases with their α -glucan substrate and the structural determinants of substrate specificity are not very well understood [5,36]. Whereas small linear maltodextrins are assumed to bind directly in the active site of phosphorylase, there is ample evidence that other substrate sites, like the glycogen storage site in rabbit-muscle phosphorylase, can be involved in

the interaction of phosphorylases with large glucan molecules such as starch or glycogen [13,14,36]. The known bacterial phosphorylases lack a glucan storage site, which could partly explain their preference of maltodextrins for polysaccharides as substrates [13]. Judging by specificity constants ($k_{\text{cat}}/K_{\text{m,glucan}}$), as well as apparent substrate binding constants, the definitely preferred substrate of *C. callunae* phosphorylase is the polysaccharide starch. Hence, the enzyme described here is the first starch phosphorylase isolated from a bacterial source. The dependence of the kinetic constants of the enzyme on the substrate size is interesting. Relative to maltodextrin phosphorylase from *E. coli* [5,11], the active site of the *C. callunae* enzyme has a 10-fold lower affinity for small oligosaccharides ($\text{DP} \leq 7$). Although two additional glucose residues in maltoheptaose did not contribute to binding relative to maltopentaose, a significant gain in binding energy was seen with much larger and branched substrates. We therefore propose a binding mode of polysaccharide in *C. callunae* phosphorylase that involves binding of the glucan molecule both at the active site with five subsites and at another site that is at least three consecutive glucose residues farther away from the active site. Since efficient binding seems to require a long linear chain portion in the substrate with $\text{DP} \geq 20$, like in starch, we believe that an additional high-affinity polysaccharide site rather than an extended active-site region is likely to exist in *C. callunae* phosphorylase. The substrate specificity of the *C. callunae* enzyme is comparable with that of the cytoplasmic forms of glucan phosphorylase from potato tuber [14] and spinach leaf [35]. Recently, a phosphorylase from *T. thermophilus* has been isolated that exhibited comparable relative activities with glycogen and maltodextrin [30]. However, the substrate preference of the thermophilic enzyme is still difficult to infer from the data available. According to k_{cat} values alone, the phosphorylase from *C. callunae* can accept various glucans with almost equal activity and, based solely on relative activities in P-mode assays, it was previously even thought to be a maltodextrin phosphorylase [15]. In contrast with the *E. coli* phosphorylase, the enzyme from *C. callunae* is very weakly inhibited by high glucan concentrations in the phosphorolysis direction. Becker et al. [12] have recently interpreted substrate inhibition in the phosphorylase reaction as the result of inhibitory binding of the sugar in the S-mode direction. We found, by comparing substrate inhibition constants with untreated or pullulanase-treated maltodextrin, that α -1,6-glycosidic linkages could play a role in determining the extent of substrate inhibition in *E. coli* phosphorylase.

Low-resolution structural characterization

As opposed to the elegant crystallographic studies on glycogen phosphorylases from rabbit muscle and yeast [34,36], the structural information on the corresponding bacterial enzymes, even at low resolution, is sparse. Here, we present a model of the solution structure of *C. callunae* phosphorylase derived from SAXS measurements. The best-fit model represented a slightly elongated, flat ellipsoid with 2-fold symmetry (rotation around the z -axis) and an axial ratio of $a/b/c = 1:(0.87\text{--}0.95):0.43$. The model yielded a theoretical scattering curve and structural parameters that agreed well with those derived experimentally. However, some deviations in the outer part of the scattering curve (in the region of $h > 2 \text{ nm}^{-1}$) are likely to reflect differences in the overall shape, specifically those of substructures, of the real phosphorylase and of the model. Earlier SAXS studies were carried out with the non-phosphorylated muscle glycogen phosphorylase *b* [41]. The molecular parameters, R_{gyr} and R_{c} , derived for the muscle enzyme dimer were 3.96 nm and 2.36 nm re-

spectively and differ slightly from those determined here with the phosphorylase from *C. callunae*. The previous study established a prismatic model of the phosphorylase *b* dimer with two shorter axes and one longer axis of the protein. The dimensions of the phosphorylase *b* derived from SAXS, 5.53:6.16:10.94 nm [41], were later found to agree well with the data of crystal analysis [37]. The model of the solution structure of *C. callunae* phosphorylase is different to the muscle enzyme in its axial ratio, as it predicted two longer axes and one shorter axis. However, the limitation of the approach by SAXS is that the form and the shape of the polypeptide in solution cannot be directly derived from the actual measurements. Hence, only model structures of the *C. callunae* phosphorylase can be proposed that result in equivalent scattering as the real enzyme molecule.

Note added in proof (received 4 August 1997)

At the time when the manuscript was submitted, the crystal structures of the *Escherichia coli* maltodextrin phosphorylase [44] and of the phosphorylase co-crystallized with maltohexaose [45] were published.

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