Substrate-binding recognition and specificity of trehalose phosphorylase from *Schizophyllum commune* examined in steady-state kinetic studies with deoxy and deoxyfluoro substrate analogues and inhibitors

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Trehalose phosphorylase is a component of the α -D-glucopyranosyl α -D-glucopyranoside (α,α -trehalose)-degrading enzyme system in fungi and it catalyses glucosyl transfer from α,α -trehalose to phosphate with net retention of the anomeric configuration. The enzyme active site has no detectable affinity for α,α -trehalose in the absence of bound phosphate and catalysis occurs from the ternary complex. To examine the role of non-covalent enzyme–substrate interactions for trehalose phosphorylase recognition, we used the purified enzyme from *Schizophyllum commune* and tested a series of incompetent structural analogues of the natural substrates and products as inhibitors of the enzyme. Equilibrium-binding constants (K_i) for deoxy- and deoxyfluoro derivatives of D-glucose show that loss of interactions with the 3-, 4- or 6-OH, but not the reactive 1- and the 2-OH, results in considerably (≥ 100 -fold) weaker affinity for sugar-binding subsite +1, revealing the requirement for hydrogen bonding with hydroxyls, away from the site of chemical transformation to position precisely the D-glucose-leaving group/nucleophile for catalysis. The high specificity of trehalose phosphorylase for the sugar aglycon during binding and conversion of O-glycosides is in contrast with the observed α -retaining

phosphorolysis of α -D-glucose-1-fluoride (α -D-Glc-1-F) since the productive bonding capability of the fluoride-leaving group with subsite +1 is minimal. The specificity constant ($19 \text{ M}^{-1} \cdot \text{s}^{-1}$) and catalytic-centre activity (0.1 s^{-1}) for the reaction with α -D-Glc-1-F are 0.10- and 0.008-fold the corresponding kinetic parameters for the enzymic reaction with α,α -trehalose. The non-selective-inhibition profile for a series of inactive α -D-glycopyranosyl phosphates shows that the driving force for the binary-complex formation lies mainly in interactions of the enzyme with the phosphate group and suggests that hydrogen bonding with hydroxyl groups at the catalytic site (subsite –1) contributes to catalysis by providing stabilization, which is specific to the transition state. Vanadate, a tight-binding phosphate mimic, inhibits the phosphorolysis of α -D-Glc-1-F by forming a ternary complex whose apparent dissociation constant of $120 \mu\text{M}$ is approx. 160-fold greater than the dissociation constant of the same inhibitor complex with α,α -trehalose.

Key words: binding energy, catalysis, catalytic subsites, glycosyl transferase.

INTRODUCTION

α -D-Glucopyranosyl α -D-glucopyranoside (α,α -trehalose) is the most widely distributed disaccharide in the vegetative cells and spores of fungi. The process of mobilization of intracellular α,α -trehalose is connected to fungal life cycle and development [1,2]. It has therefore attracted much interest in regulation and mechanism of action of α,α -trehalose-converting enzymes in general and trehalase in particular [1,3–5]. Recent studies have revealed clearly the involvement of another enzyme trehalose phosphorylase in catabolism of α,α -trehalose in fungi [2,6,7]. Trehalose phosphorylases (EC 2.4.1.64) catalyse degradation of α,α -trehalose with net retention of anomeric configuration and by using phosphate as the ultimate glucosyl acceptor [8]. Hence, α -D-glucose-1-phosphate (Glc-1-P) and D-glucose are the reaction products. Trehalose phosphorylases are glucosyl transferases which, in spite of the presence of 55 M water, synthesize glycosidic bonds without the occurrence of detectable error hydrolysis [6,7]. Based on similarity at the level of primary structure, trehalose phosphorylases from *Grifola frondosa* (GenBank® accession no. AB010105) [9] and *Pleurotus sajor-caju* (GenBank® accession no. AF149777) have been classified into Family GT4 of the glycosyl transferase families (<http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>) [10]. The enzyme from the basidiomycete *Schizophyllum commune* has been characterized biochemically

[6,7,11] and has been shown by peptide mass mapping to share close structural similarity with the classified trehalose phosphorylase sequences [7]. It utilizes a steady-state ordered Bi Bi kinetic mechanism as shown in Scheme 1. The active site of trehalose phosphorylase contains two separate binding sites for the substrates phosphate and α,α -trehalose. The α,α -trehalose-binding site is composed of two subsites, each of which interacts with one constituent glucosyl residue of the disaccharide substrate [7,11]. Ternary-complex conversion and release of Glc-1-P are major rate-limiting steps for the phosphorolysis direction of the reaction [7].

A fundamental catalytic principle for enzymes is that they make use of binding interactions with groups of the substrate not

Scheme 1 Kinetic mechanism of trehalose phosphorylase from *S. commune* [7]

Abbreviations used: Glc-1-P, α -D-glucose-1-phosphate; α -D-Glc-1-F, α -D-glucose-1-fluoride; α,α -trehalose, α -D-glucopyranosyl α -D-glucopyranoside.

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directly involved in the chemical transformation for binding recognition and transition-state stabilization [12]. We are interested in providing an explanation for the observed high specificity of trehalose phosphorylase [8,11] at a molecular level by determining how non-covalent enzyme interactions define the energetic profile for the transitory and central complexes and the transition state of glucosyl transfer. The carbohydrate nature of α,α -trehalose would imply an essential role of the sugar hydroxyl groups for bonding. Replacement of a hydroxyl by hydrogen or fluorine has a strong disruptive effect on hydrogen bonds originally present at this position, but is otherwise of a structurally conservative nature (see [13] for a detailed discussion). Therefore a systematic substrate variation approach ($\text{OH} \rightarrow \text{H}$, $\text{OH} \rightarrow \text{F}$) in which the consequences of the respective substitution are analysed in kinetic and inhibitor-binding studies has the potential to explore selectively the hydrogen-bonding interactions between a carbohydrate-active enzyme and an individual hydroxyl group of the substrate at different points of the reaction co-ordinate [13,14].

The present paper evaluates a series of deoxy and deoxyfluoro derivatives of D-glucose and Glc-1-P, and some other close structural analogues of these sugars as alternative substrates of the reverse-enzymic glucosyl transfer catalysed by trehalose phosphorylase from *Schizophyllum commune*. We report a detailed equilibrium-binding study in which incompetent analogues of the natural glucosyl donors and acceptors were used as inhibitors of the enzyme and their affinities for sugar-binding subsite +1 and the catalytic subsite (–1) [15] were measured. Furthermore, we report kinetic parameters for the reactions of trehalose phosphorylase with α -D-glucose-1-fluoride (α -D-Glc-1-F) and arsenate which serve as alternative substrates replacing α,α -trehalose and phosphate, respectively. α -D-Glc-1-F was an interesting ‘truncated’ substrate for phosphorolysis [8] because as opposed to the sugar aglycon in the canonical substrate α,α -trehalose, the fluoride-leaving group cannot interact strongly with enzyme functional groups at subsite +1 (see [16] for a recent review of glycosyl fluorides as enzyme substrates). The inhibition of the enzymic phosphorolysis of α -D-Glc-1-F by the tightly binding inhibitor vanadate [11] was characterized by equilibrium-binding analysis.

EXPERIMENTAL

Materials and assays

Purified trehalose phosphorylase from *S. commune* (strain BT 2115) was prepared from fresh fungal mycelium obtained by growing the organism for 48 h on D-glucose as major carbon source [6]. The materials and assays used are as described elsewhere [6].

Initial-rate studies

All experiments were carried out at 30 °C in 20 mM MES buffer, pH 6.6, containing 2 mM EDTA and 2 mM β -mercaptoethanol. Initial rates in forward- and reverse-enzymic reactions were determined using discontinuous assays and measuring the release of Glc-1-P and phosphate, respectively [6].

Unless mentioned otherwise, inhibition studies were carried out at three or four constant concentrations of the inhibitor, corresponding to the range approx. 1–5 times the respective apparent K_i value. The initial rates in the presence of inhibitor were recorded under conditions in which the concentration of one substrate was varied and the other substrate was present at a constant, saturating concentration. In separate controls, it was

proved that none of the used inhibitor functioned as a substrate of trehalose phosphorylase. The limit of detection in these assays corresponded to a reactivity approx. 5×10^{-5} -fold lower than that of the natural substrates.

Double-inhibition studies were carried out to determine whether two inhibitors of trehalose phosphorylase, α -D-mannose 1-phosphate and 1,5-anhydroglucitol, bind either to the enzyme active site independently or synergistically, or are mutually exclusive in their binding. Initial rates were recorded under conditions that used α,α -trehalose and phosphate in constant non-saturating concentrations of 40 and 10 mM respectively and several different levels of each inhibitor, whereby one inhibitor was used at a constant concentration and the other inhibitor's concentration was varied. The experimental reaction rates were determined from the concentration of Glc-1-P released as a function of time and analysed graphically.

Enzymic reactions with glucosyl fluorides

The α - and β -anomers of D-glucose-1-fluoride were tested as possible glucosyl donors of the forward and reverse reactions catalysed by trehalose phosphorylase. The conditions described above were used. When the conventional assays [6] would have failed to detect product formation, e.g. the synthesis of α,α -trehalose from α -D-Glc-1-F and D-glucose, substrate depletion was monitored and TLC of samples taken at certain times during the enzymic reactions was used to confirm that substrate conversion occurred synchronously with the formation of the expected product. The extent to which the spontaneous hydrolysis of α - or β -D-Glc-1-F might interfere with the determination of enzyme activity under the respective reaction conditions was assessed in separate control experiments lacking the enzyme and D-glucose. The non-catalysed formation of D-glucose from each fluoride (40 mM) was monitored by TLC and by using an enzymic assay based on the glucose oxidase-catalysed reaction [6]. Decomposition of the fluorides is not a critical factor to be considered during initial-rate measurements but must be taken into account when reaction times of 24–48 h are necessary to detect product formation. The fluorides were tested as inhibitors of the forward and reverse reactions catalysed by trehalose phosphorylase, wherever possible.

Enzymic reaction with arsenate and in the presence of vanadate

When arsenate replaced phosphate as glucosyl acceptor in the reaction of trehalose phosphorylase with α,α -trehalose, initial rates were determined in discontinuous assays and obtained from the linear increases of D-glucose concentration released versus the reaction time. Since arsenate is known to react with sugar hydroxyl groups to give esters [17,18], the reactants were mixed immediately before the enzymic reaction was started by the addition of the phosphorylase. Precautions were taken when vanadate was used as inhibitor of trehalose phosphorylase as reported previously [19].

Data processing and analysis

A preliminary data analysis was performed by plotting reciprocal initial velocities v against reciprocal substrate concentrations A . The experimental initial rates were then fitted to the appropriate equation by using the unweighed least-squares method and the SigmaPlot 2000 program for Windows (SPSS, Chicago, IL, U.S.A.):

$$v = k_{\text{cat}} EA / (K + A) \quad (1)$$

$$v = k_{\text{cat}} EA/[K(1 + I/K_{\text{is}}) + A] \quad (2)$$

$$v = k_{\text{cat}} EA/[K(1 + I/K_{\text{is}}) + A(1 + I/K_{\text{ii}})] \quad (3)$$

Linear double-reciprocal plots were fitted to eqn (1), where k_{cat} is the catalytic-centre activity, E the enzyme concentration based on a molecular mass of 61 kDa and K an apparent Michaelis constant. Equations (2) and (3) were used when linear competitive inhibition and linear mixed-type inhibition were observed, respectively. K_{is} and K_{ii} relate to an inhibitor I that affects the slope and the intercept of the double-reciprocal plot, respectively. Unless otherwise mentioned, the correlation coefficient for non-linear regression was 0.98 or higher, and problems with linearly correlated parameters were not encountered.

RESULTS AND DISCUSSION

Evaluation of glucosyl fluorides as alternative glucosyl donors of the forward enzymic reaction

Kinetic parameters for the enzymic reaction with α -D-Glc-1-F were obtained from a nonlinear fit of eqn (1) to initial-rate data measured in the presence of a saturating concentration of phosphate (50 mM): $k_{\text{cat}} = 0.11 \pm 0.03 \text{ s}^{-1}$; $K_{\text{m}} = 5.9 \pm 1.3 \text{ mM}$; $k_{\text{cat}}/K_{\text{m}} = 19 \text{ M}^{-1} \cdot \text{s}^{-1}$. There is an approx. 10-fold increase in specificity of trehalose phosphorylase for its reaction with α, α -trehalose ($k_{\text{cat}}/K_{\text{trehalose}} = 187 \text{ M}^{-1} \cdot \text{s}^{-1}$) when compared with its reaction to α -D-Glc-1-F. The corresponding k_{cat} values differ by 120-fold. Depending on the extent to which the overall chemistry is rate-limiting for the enzymic reaction, a decrease in $k_{\text{cat}}/K_{\text{m}}$ observed across a series of different substrates is expected to produce an effect in the corresponding k_{cat} value which may be of the same or a smaller magnitude but usually not greater. A possible explanation is that α -D-Glc-1-F binds to enzyme · phosphate complex in two different ways, one of which is productive and takes place at the sugar-binding subsite -1 and another at subsite +1 which is non-productive and leads to an inactive enzyme · substrate complex. (Inhibition studies discussed below reveal that α -D-Glc-1-F binds to subsite +1 in the trehalose phosphorylase · Glc-1-P complex.) The observed effective catalytic-centre activity will be decreased by the fraction of productively bound substrate F, whereas $k_{\text{cat}}/K_{\text{m}}$ will not be affected by non-productive binding. An estimate of 0.08 (= 10/120) is obtained for F or, in other words, approx. 8% of α -D-Glc-1-F bound to trehalose phosphorylase appears to interact productively with the enzyme.

β -D-Glc-1-F is not phosphorolysed noticeably by the enzyme. 2-Deoxy-2-fluoro- α -D-Glc-1-F is not a substrate of trehalose phosphorylase but inhibits the enzymic reaction with α, α -trehalose. Inductive effects of the fluorine at C-2 might explain the observed lack of reactivity. A K_{is} value of $21 \pm 10 \text{ mM}$ was determined from a nonlinear fit of eqn (2) to initial rates obtained with α, α -trehalose as the variable substrate, whereas phosphate concentration was constant and saturating at 40 mM. α -D-Glc-1-F behaves as a competitive inhibitor against α, α -trehalose in the phosphorolysis direction, as expected for an analogue of the second substrate in an ordered Bi Bi kinetic mechanism (Scheme 1). The apparent K_{is} for α -D-Glc-1-F is 8.2 mM and approximates the Michaelis constant for the fluoride, in agreement with the theoretical equality of both parameters [20].

In the absence or presence of phosphate, trehalose phosphorylase did not catalyse the hydrolysis of α -D-Glc-1-F within the limit of detection in our experimental assay (0.1%). Therefore the occupancy of sugar-binding subsite +1 of the enzyme is apparently not a requirement for glucosyl transfer to take place without the occurrence of significant 'error' in hydrolysis.

Figure 1 Inhibition of the enzymic phosphorolysis of α -D-Glc-1-F by vanadate

Initial rates were recorded by varying the concentration of α -D-Glc-1-F and keeping the concentration of phosphate constant at 50 mM: ●, 0 mM; □, 0.05 mM; ▲, 0.5 mM; ◇, 5.5 mM vanadate.

Figure 2 Model of the inhibition of trehalose phosphorylase by vanadate in the reactions with α, α -trehalose (A) and α -D-Glc-1-F (B,C)

The fluoride could bind, in principle, at subsite -1 (B) and +1 (C), and either of the binding modes is compatible with the observed inhibition. The arrows show probable hydrogen bonds and the polarities of these bonds.

Inhibition of the phosphorolysis of α -D-Glc-1-F by vanadate

Initial rates of phosphorolysis of α -D-Glc-1-F were recorded in the presence of three different concentrations of vanadate, whereas the concentration of α -D-Glc-1-F was varied and phosphate concentration was constant at 50 mM. Figure 1 shows a double-reciprocal plot of the results. The slopes in Figure 1 increase with increasing vanadate concentration and their intersection point is on the abscissa or slightly to the left of it. Assuming that a ternary complex enzyme · vanadate · α -D-Glc-1-F is formed via rapid-equilibrium-ordered binding [11], vanadate is expected to behave as a mixed-type inhibitor with respect to α -D-Glc-1-F. Plots of $1/v$ versus $1/[\alpha$ -D-Glc-1-F] should be nonlinear at high substrate concentration. However, the observed

Table 1 Dissociation constants of analogues of D-glucose that bind to subsite +1 of the trehalose phosphorylase · Glc-1-P binary complex

Inhibitor	K_i (mM)*
D-Glucose	1.4†
Deoxy-D-glucose derivatives 3-OH → H; 3-OH → F; 4-OH → H; 4-OH → F; 6-OH → H; 6-OH → F	≥ 100‡
D-Xylose	1200 ± 400
D-Galactose	1000 ± 300
α-D-Glc-1-F	5.6 ± 0.7
2-Deoxy-2-F-α-D-Glc-1-F	40 ± 8
1,5-Anhydroglucitol	0.72 ± 0.10§
D-Glucal	0.32 ± 0.04§
2-Deoxy-2-amino-D-Glc (D-glucosamine)	46 ± 7

* Determined by fitting eqn (2) to initial-rate data recorded at 30 °C and pH 6.6 and obtained under conditions where several constant concentrations of the inhibitor were present and D-glucose concentration was varied, whereas Glc-1-P concentration was constant at 40 mM and saturating.

† Calculated from steady-state kinetic parameters in ref. [7].

‡ Less than 10% inhibition occurred for [I] in the range between 10 and 100 mM.

§ Taken from ref. [7].

K_{is} and K_{ii} values will contain the factors $K_{ip_1}/[P_1]$ (= 0.5) and $K_{p_1}/[P_1]$ (= 0.028) [7], respectively. Therefore the intercept effect of the inhibition (K_{ii}) as well as the curvature of the lines in the double-reciprocal plot may be difficult to detect. A nonlinear fit of the data to eqn (3) provided a K_{is} value of $123 \pm 33 \mu\text{M}$ for vanadate. Vanadate thus inhibits the phosphorolysis of α-D-Glc-1-F approx. 160 times more weakly than the analogous reaction with α,α-trehalose ($K_{is} = 0.75 \mu\text{M}$ [11]). The decrease in the differential binding energy stabilizing the ternary complex ($\Delta\Delta G^\circ = RT \ln 160 = 12.8 \text{ kJ/mol}$) probably reflects a weakened hydrogen bond, H → F instead of H → O, in the α-D-Glc-1-F · vanadate adduct, compared with the corresponding α,α-trehalose adduct [11] and perhaps the absence of interactions at subsite +1. However, our data do not reveal at which subsite of trehalose phosphorylase, α-D-Glc-1-F binds to form the adduct with vanadate (Figure 2).

Utilization of arsenate

Initial rates for arsenolysis of α,α-trehalose were determined by using arsenate as the varied substrate, whereas the concentration of α,α-trehalose was constant and saturating (300 mM). The kinetic parameters $k_{cat} = 2.0 \pm 0.1 \text{ s}^{-1}$, $K_{arsenate} = 3.8 \pm 0.4 \text{ mM}$ and $k_{cat}/K_{arsenate} = 526 \text{ M}^{-1} \cdot \text{s}^{-1}$ were obtained by fitting eqn (1) to the experimental data. There is an approx. 18-fold increase in specificity of α,α-trehalose for its reaction with phosphate, compared with its reaction to arsenate.

Substrate-binding recognition at sugar-binding subsite +1

A series of inactive structural analogues of D-glucose were tested as inhibitors of trehalose phosphorylase to determine their binding to subsite +1 of the enzyme. Results are summarized in Table 1, where K_i values for the inhibitors are compared with the dissociation constant of D-glucose. Derivatives of D-glucose carrying a replacement OH → F or OH → H at C-3, C-4 or C-6 do not show measurable binding with the enzyme · Glc-1-P complex when used in concentrations of up to 100 mM. The K_i values for these compounds must therefore be much greater than 100 mM. The inhibition ($K_i \approx 1 \text{ M}$) of trehalose phosphorylase by D-galactose and D-xylose confirmed that close structural

Scheme 2 Two-step-binding mechanism of D-glucose to form the productive ternary complex with trehalose phosphorylase and Glc-1-P

E_{bin} is the binary complex of enzyme and Glc-1-P. The broken arrow indicates the probable effect on binding due to the loss of hydrogen-bonding interactions with non-reacting hydroxyl groups of D-glucose, i.e. a decrease in the stability of ${}^*E_{bin} \cdot Glc$ by increasing k_{off} . K_d is the dissociation constant of the initial encounter complex and the observed K_i values are related to microscopic rate constants by the expression $K_d/(1 + k_{on}/k_{off})$.

analogues of D-glucose are capable of binding with the binary complex, however, very weakly.

In the absence of interactions other than those at the OH at C-3, C-4 or C-6, water would out-compete D-glucose because water is present in large excess (55 M) and the capabilities to function as a donor or acceptor of a hydrogen for bonding are similar for the sugar hydroxyl groups ($pK_a \approx 14$ –16) and water ($pK_a \approx 16$). For example, the ≥ 71-fold (= 100/1.4) stronger binding of D-glucose relative to 3-deoxy-D-glucose, indicates a ≥ 71-fold preference of the functional groups on the enzyme to interact with the 3-OH of D-glucose than with 55 M water when the deoxy analogue is bound. It leads to an estimate of ≥ 3905 M (= 71 × 55) for the effective concentration of the 3-OH and similarly the effective concentrations of the 4-OH and 6-OH when bound to trehalose phosphorylase, relative to the OH of water. Positioning of D-glucose so that it interferes with the ability of bulk water to hydrogen bond with the active-site functional groups, is expected to contribute to the value of the effective concentration of each OH group.

The small catalytic efficiency of trehalose phosphorylase for the enzymic reaction with D-glucose ($260 \text{ M}^{-1} \cdot \text{s}^{-1}$ [7]) suggests that binding of D-glucose with the enzyme · Glc-1-P complex takes place in two steps whereby a rapid-equilibrium formation of an initial weak encounter complex is followed by a slower conformational rearrangement of the active site to give the productive ternary complex. We envisage that the OH groups at C-3, C-4 and C-6 facilitate the conformational step by stabilizing specifically the productive complex, probably by non-covalent interactions in which each OH functions as a donor of a hydrogen for bonding with the enzyme (Scheme 2). This stabilization would lead to a decrease in the dissociation constant of the encounter complex by a factor of approx. 700 ($\approx K_{i, galactose}/K_{i, glucose}$; cf. Table 1). In such a situation, a D-glucose-dependent conformational change in protein structure would play an essential role to activate the enzyme.

The relationship $\Delta\Delta G^\circ = RT \ln(K_{i,A}/K_{i,B})$ was used to calculate the differential binding energy that stabilizes the ground-state ternary complex of enzyme · Glc-1-P with inhibitor A, relative to the corresponding complex with another inhibitor B. The replacement OH → F at C-1 leads to weakened binding (D-glucose versus α-D-Glc-1-F; $\Delta\Delta G^\circ = 3.5 \text{ kJ/mol}$) as does the same substitution at C-2 (α-D-Glc-1-F versus 2-deoxy-2-fluoro α-D-Glc-1-F; $\Delta\Delta G^\circ = 5.0 \text{ kJ/mol}$). The replacement of the 1-OH by H has no effect on binding, as seen from a comparison of the dissociation constants for D-glucose and 1,5-anhydroglucitol ($\Delta\Delta G^\circ = 1.7 \text{ kJ/mol}$). The relatively small K_i value for D-glucal indicates that the 2-OH and the preferred chair conformation of D-glucose are not required for binding with the enzyme · Glc-1-P complex. Note that the endocyclic double bond in D-glucal favours a half-chair conformation of this compound.

Table 2 Dissociation constants of analogues of Glc-1-P that bind to subsite — 1 of trehalose phosphorylase

Compound	K_i (mM)*
Glc-1-P	2.4†
2-Deoxy-2-F Glc-1-P	10.6 ± 2.8
α -D-Mannose 1-P	1.60 ± 0.15
α -D-Galactose 1-P	11.5 ± 1.2
α -D-Xylose 1-P	11.0 ± 3.0
α -D-Glucuronic acid 1-P	13.4 ± 2.1
β -D-Glucose 1-P	N.D.†
2-Deoxy-2-F- α -D-glucose 1-F	N.D.‡

* Determined by fitting eqn (2) to initial-rate data recorded at 30 °C and pH 6.6 and obtained under conditions where several constant concentrations of the inhibitor were present and Glc-1-P concentration was varied, whereas D-glucose concentration was constant at 1 M and saturating.

† From ref. [7].

‡ No detectable inhibition for [I] in the range between 10 and 100 mM.

Figure 3 Yonetani–Theorell plot for the inhibition of trehalose phosphorylase by α -D-mannose 1-phosphate and 1,5-anhydroglucitol

The experiment was conducted at four different concentrations of 1,5-anhydroglucitol. ●, 0.0 mM; ○, 1.0 mM; ▲, 2.0 mM; △, 4.0 mM. Reaction rates were recorded in the direction of disaccharide phosphorylase using 80 mM α,α -trehalose and 10 mM phosphate, measuring the formation of Glc-1-P.

Substrate-binding recognition at sugar-binding subsite — 1

Binding of inactive derivatives of Glc-1-P to the subsite — 1 of trehalose phosphorylase was studied in inhibition experiments in which Glc-1-P concentration varied and D-glucose concentration was constant and saturating (1 M). All derivatives were competitive inhibitors with respect to Glc-1-P. K_{is} values were obtained from nonlinear fits of the data to eqn (2) and are summarized in Table 2. They reveal modest decreases in binding affinity in response to structural alterations of Glc-1-P.

Analogues of Glc-1-P may function as dead-end inhibitors of the enzyme, in which case abortive binary complexes will be formed which cannot add D-glucose or a derivative thereof. Alternatively, if binding of sugar 1-phosphates induces trehalose phosphorylase in the proper conformational state that permits D-glucose to bind, a catalytically inactive ternary complex will be formed which can regenerate the free enzyme only by dissociation. Double-inhibition experiments were carried out in an effort to

address this question. A Yonetani–Theorell plot [21] for the inhibition of the enzymic phosphorylase of α,α -trehalose by α -D-mannose 1-phosphate and 1,5-anhydroglucitol is shown in Figure 3. The results reveal a parallel-line pattern, indicating that neither inhibitor binds to the enzyme active site at the same time. The effect of α -D-mannose 1-phosphate was as expected from the corresponding K_i value shown in Table 2. These results show that the sugar 1-phosphate behaves as a dead-end inhibitor of the enzyme and unlike Glc-1-P, is not capable of inducing affinity for 1,5-anhydroglucitol at subsite +1 (see Table 1). We have no explanation, however, for the specificity of 1,5-anhydroglucitol for binding to the complexes of the enzyme with Glc-1-P and phosphate but not the complex with α -D-mannose 1-phosphate.

Implications for the enzymic mechanism of trehalose phosphorylase

Binding recognition of the disaccharide substrate by trehalose phosphorylase is dependent strongly on hydrogen-bonding interactions at subsite +1 whereby parts of the 1-O-D-glucosyl leaving group of α,α -trehalose that are relatively distant from the reactive centre contribute most to binding affinity. A conformational change in protein structure probably accompanies substrate binding at subsite +1 and may aid in the correct alignment of substrates and enzyme catalytic groups in the ternary complex. Probably, it is the major source of the large aglycon specificity of trehalose phosphorylase. Interestingly, recognition of trehalose phosphorylase and trehalase [22] may show a very similar aglycon dependence. Both enzymes can tolerate loss of interactions with the 2'-OH [5] but are otherwise specific for the *gluco* configuration of the sugar binding to subsite +1.

Considering a leaving group-dependent 'activation' of trehalose phosphorylase for O-glucoside bond cleavage, the ability of the enzyme to catalyse the phosphorylase of α -D-Glc-1-F is interesting as this reaction must proceed necessarily without contributions to the transition-state stabilization derived from non-covalent interactions with the departing fluoride at subsite +1. It is noteworthy, however, that α -methyl D-glucoside, capability of productive bonding with subsite +1 of which is impaired severely when compared with 1-O-D-glucose in α,α -trehalose is not a substrate of trehalose phosphorylase and does not show detectable binding to the enzyme phosphate complex. This emphasizes the possible and not completely unexpected differences in the enzymic mechanisms of C–F and C–O bond cleavage by trehalose phosphorylase.

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