

# $\alpha$ -Retaining glucosyl transfer catalysed by trehalose phosphorylase from *Schizophyllum commune*: mechanistic evidence obtained from steady-state kinetic studies with substrate analogues and inhibitors

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Fungal trehalose phosphorylase is classified as a family 4 glucosyltransferase that catalyses the reversible phosphorolysis of  $\alpha,\alpha$ -trehalose with net retention of anomeric configuration. Glucosyl transfer to and from phosphate takes place by the partly rate-limiting interconversion of ternary enzyme–substrate complexes formed from binary enzyme–phosphate and enzyme– $\alpha$ -D-glucopyranosyl phosphate adducts respectively. To advance a model of the chemical mechanism of trehalose phosphorylase, we performed a steady-state kinetic study with the purified enzyme from the basidiomycete fungus *Schizophyllum commune* by using alternative substrates, inhibitors and combinations thereof in pairs as specific probes of substrate-binding recognition and transition-state structure. Orthovanadate is a competitive inhibitor against phosphate and  $\alpha$ -D-glucopyranosyl phosphate, and binds  $3 \times 10^4$ -fold tighter ( $K_i \approx 1 \mu\text{M}$ ) than phosphate. Structural alterations of D-glucose at C-2 and O-5 are tolerated by the enzyme at subsite +1. They lead to parallel effects of approximately the same magnitude (slope = 1.14;  $r^2 = 0.98$ ) on the

reciprocal catalytic efficiency for reverse glucosyl transfer [ $\log(K_m/k_{\text{cat}})$ ] and the apparent affinity of orthovanadate determined in the presence of the respective glucosyl acceptor ( $\log K_i$ ). An adduct of orthovanadate and the nucleophile/leaving group bound at subsite +1 is therefore the true inhibitor and displays partial transition state analogy. Isofagomine binds to subsite –1 in the enzyme–phosphate complex with a dissociation constant of  $56 \mu\text{M}$  and inhibits trehalose phosphorylase at least 20-fold better than 1-deoxynojirimycin. The specificity of the reversible azasugars inhibitors would be explained if a positive charge developed on C-1 rather than O-5 in the proposed glucosyl cation-like transition state of the reaction. The results are discussed in the context of  $\alpha$ -retaining glucosyltransferase mechanisms that occur with and without a  $\beta$ -glucosyl enzyme intermediate.

**Key words:** family 4, glycosyltransferase, intermediate, retaining mechanism.

## INTRODUCTION

Glycosylhydrolases (GHs) catalyse the hydrolysis of glycoside substrates with one of two possible stereochemical outcomes: retention or inversion of configuration at the anomeric centre. The retaining GHs use a two-step (double-displacement) mechanism that involves a configurationally inverted glycosyl-enzyme intermediate [1–4]. Catalysis is promoted by a pair of carboxylic acids, one of which functions as the nucleophile and the other as the general acid–base providing catalytic assistance to glycosidic bond cleavage and the nucleophilic addition of water [1,4]. The transition states leading to and from the covalent intermediate have substantial oxocarbenium-ion character [1].

Unlike GHs, glycosyltransferases (GTs) catalyse the synthesis of glycosidic bonds in physiology. They have been classified on the basis of similarities at the level of primary structure [5,6]; currently 52 potential GT families have been identified (comprehensively summarized at <http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>). Like GHs, GTs can be also classified depending on whether they retain the anomeric configuration of the glycosyl donor in the glycoside product or invert it. The stereochemistry of the enzymic synthesis is believed to be a constant trait within a given GT family [1].

The reactions catalysed by GHs and GTs are thought to pass through common glycosyl-cation-like transition states [1,7,8]. Considerable mechanistic similarities are therefore expected to exist in both enzyme classes. However, the enzymic machinery

employed by GTs to provide stabilization of the transition state might be different from that used by GHs. Catalytic carboxy groups, omnipresent in GHs, have been located in the X-ray structures of several inverting [7,9–12] and retaining [13] GTs but seem to be absent from others [14–17]. What these GT structures also show is a mechanistic diversity apparently lacking in GHs and arguably reflecting the special characteristic of each individual GT.

The catalytic mechanism used by retaining GTs is not very well understood. Precedent with GHs would strongly favour a covalent intermediate but attempts to demonstrate its formation have not been fruitful in several cases (see, for example, [18,19]). The X-ray structures of glycogen phosphorylase [16] (GT family 35) and  $\alpha$ 1,4-galactosyltransferase [17] (GT family 8) have revealed the absence of a carboxylate group appropriately positioned to be a candidate nucleophile. By contrast, the structure of the complex of bovine  $\alpha$ 1,3-galactosyltransferase (GT family 6) and UDP- $\alpha$ -D-galactose provided evidence of the formation of a  $\beta$ -galactosyl intermediate [13] in which the sugar seemed to be linked to a catalytic Asp residue. With a glycosyl-enzyme intermediate being elusive in glycogen phosphorylase and  $\alpha$ 1,4-galactosyltransferase, alternative models have been proposed in an effort to explain the retention of configuration [1,16,17]. Among them is the so-called internal return ( $S_Ni$ ) mechanism, which has been discussed in the context of non-catalysed solvolysis reactions of glucopyranosides [20] and the enzymic glucosyl transfer [16,17]. It assumes the approach of

Abbreviations used: GH, glycosylhydrolase; Glc1P;  $\alpha$ -D-glucopyranosyl phosphate; GT, glycosyltransferase;  $S_Ni$ , internal return.

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the nucleophile towards the reactive anomeric carbon from the same side as the leaving group departs from. Predicted general features of the  $S_Ni$  mechanism are therefore (1) the development of interactions, for example a hydrogen bond [20], between the leaving group and the nucleophile in the transition state; and (2) a major electrostatic stabilization of the oxocarbenium ion taking place from the 'front' side, not from the 'back' side where an enzyme nucleophile would be positioned. In this model, internal return would occur from within the ion pair by the recombination of C-1 and the oxygen atom of the nucleophile. The experimental evidence supporting the  $S_Ni$  mechanism of enzymic glycosyl transfer is sparse, which might be due to the difficulty of designing experiments capable of unambiguously proving or disproving internal return [1].

Fungal trehalose phosphorylase is an  $\alpha$ -retaining, family 4 GT. It catalyses the reversible phosphorolysis of  $\alpha,\alpha$ -trehalose into  $\alpha$ -D-glucose and  $\alpha$ -D-glucopyranosyl phosphate (Glc1P) [21]. The enzyme from the basidiomycete fungus *Schizophyllum commune* follows a steady-state ordered kinetic mechanism with binary enzyme-phosphate and enzyme-Glc1P complexes [19]. The mechanism implies the binding of  $\alpha,\alpha$ -trehalose or  $\alpha$ -D-glucose before ternary complex interconversion occurs; this might proceed with or without the requirement for a covalent intermediate [19].

Here we report a detailed steady-state kinetic study of glycosyl transfer to and from phosphate catalysed by trehalose phosphorylase from *S. commune* by using substrate analogues, inhibitors and combinations thereof in pairs. The results emphasize an essential role of the enzyme-bound phosphate in catalysis and provide a basic scheme of the enzymic mechanism. They are compatible with the expected double-inversion pathway or the unusual  $S_Ni$  reaction.

## EXPERIMENTAL

### Materials and assays

Trehalose phosphorylase from *S. commune* (strain BT 2115) was produced and purified to apparent electrophoretic homogeneity by using procedures reported recently [22]. 2-Deoxy-D-glucose, 3-deoxy-D-glucose, 4-deoxy-D-glucose and 6-deoxy-D-glucose were from Chemprosa (Graz, Austria). Analogues of D-glucose carrying a substitution of F for OH at positions 2, 3, 4 or 6 were synthesized by reported methods [23] and kindly provided by Dr M. Albert and Dr K. Dax (Institute of Organic Chemistry, Technical University of Graz, Graz, Austria). Isofagomine and 1-deoxynojirimycin (as the adduct with chloride) were gifts from Dr I. Lundt (Institute of Organic Chemistry, Technical University of Lyngby, Lyngby, Denmark) and Dr A. Stütz (Institute of Organic Chemistry, Technical University of Graz, Graz, Austria) respectively.  $\alpha,\alpha$ -Thiotrehalose was generously provided by Dr H. Driguez (CERMAV Grenoble, Grenoble, France). The enzyme assays used were described elsewhere [21]. D-arabino-Hexosulose ('D-glucosone'; '2-keto-D-glucose') was prepared with fungal pyranose 2-oxidase [24]. It was shown with the use of TLC, HPLC and a glucose oxidase-based enzymic assay that 2-keto-D-glucose did not contain detectable contamination by D-glucose (0.5% or less).

### Enzymic reactions with alternative glycosyl acceptors

Screening of alternative glycosyl acceptors was performed for the reverse reaction catalysed by trehalose phosphorylase. This was done at 30 °C in 20 mM Mes (sodium salt) buffer, pH 6.6, containing 2 mM EDTA and 2 mM 2-mercaptoethanol. The glycosyl acceptor and Glc1P were used at a constant con-

centration of 40 mM, with a total reaction volume of 300  $\mu$ l. They were incubated in the presence of enzyme (approx. 1 unit/ml) for 24 h. TLC with appropriate carbohydrate standards was used to monitor product formation and substrate depletion. The release of phosphate from Glc1P was also measured. The analogues with which enzyme activity was clearly detectable were used for further kinetic studies.

### Initial velocity studies

All experiments were performed at 30 °C in 20 mM Mes buffer, pH 6.6, containing 2 mM EDTA and 2 mM 2-mercaptoethanol. Initial velocities in the forward and reverse reactions were determined by using discontinuous assays under conditions reported recently and by measuring the release of Glc1P and phosphate respectively [22].

Unless mentioned otherwise, inhibition studies with a single inhibitor were performed at four constant concentrations of the inhibitor, covering the range of between 1-fold and 5-fold the respective  $K_i$  values. Initial velocities were recorded under conditions in which one substrate was varied and the other substrate was present at a constant, saturating, concentration. Solutions of D-glucono- $\delta$ -lactone were prepared immediately before use. 1-Deoxynojirimycin was incubated in buffer at 4 °C for 24 h to permit the dissociation of any adduct that might have been present after its dissolution. Free (protonated) 1-deoxynojirimycin is thought to be the actual inhibitor.

Double-inhibition studies were performed to determine whether two inhibitors of trehalose phosphorylase bind independently or synergistically to the enzyme's active site or whether they are mutually exclusive in their binding. The initial velocities were measured under conditions that used both substrates of the forward or reverse enzymic reactions at constant non-saturating concentrations (40 mM D-glucose and 10 mM Glc1P; 80 mM  $\alpha,\alpha$ -trehalose and 10 mM P<sub>i</sub>). Data were recorded at four or five different levels of each inhibitor; one inhibitor was used at a constant concentration and that of the other inhibitor was varied. The experimental reaction rates were analysed graphically by using the Yonentani-Theorell plot [25], which is a plot of reciprocal initial velocity against inhibitor concentration.

### Data processing

A preliminary data analysis was performed by plotting the reciprocal of initial velocity ( $v$ ) against the reciprocal of substrate concentration ( $A$ ). The experimental initial rates were then fitted to the appropriate one of the following equations by using the least-squares method and the Sigmaplot 2000 program (SPSS, Chicago, IL, U.S.A.):

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

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

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

Linear double-reciprocal plots were fitted to eqn (1), where  $k_{\text{cat}}$  is the turnover number,  $E$  is the molar enzyme concentration based on a molecular mass of 61 kDa for trehalose phosphorylase, and  $K_m$  is an apparent Michaelis constant. Eqn (2) or (3) was used when linear competitive or uncompetitive inhibition was observed respectively.  $K_{\text{is}}$  and  $K_{\text{ii}}$  relate to inhibitors that affect the slope and intercept of the double reciprocal plot respectively;  $I$  is the inhibitor concentration. Unless mentioned otherwise, the correlation coefficient for non-linear regression was 0.98 or greater, and problems with linearly correlated parameters were not encountered.

### Enzymic reactions in the presence of vanadate

A stock solution of 20 mM NaH<sub>2</sub>VO<sub>4</sub> was prepared by dissolving V<sub>2</sub>O<sub>5</sub> in 1 M NaOH and diluting to volume. HCl (1 M) was used to adjust the pH to 7.0. The yellow colour of the solution indicated that, apart from the probable active monomeric vanadate, oligomeric forms of vanadate were also present. The colour disappeared completely on dilution to approx. 0.1 mM. To minimize the formation of esters of vanadate and sugars [26], all reactants were mixed immediately before the enzymic reaction was started by the addition of enzyme. Initial velocities were measured as described above. Superoxide dismutase (50 units/ml) was added during the enzymic determination of Glc1P to avoid possible interference by the superoxide radical [27].

## RESULTS AND DISCUSSION

### Alternative glucosyl acceptors in the reverse enzymic reaction

A complete series of analogues of D-glucose in which an individual hydroxy group had been replaced by hydrogen or fluorine were evaluated as alternative glucosyl acceptors of the reverse reaction catalysed by trehalose phosphorylase. In addition, 5-thio-D-glucose, 2-keto-D-glucose, D-mannose and 2-amino-2-deoxy-D-glucose were also tested as possible substrates of the enzyme. Table 1 reports kinetic parameters for the enzymic reaction with the compounds whose capability of acting as glucosyl acceptor in place of D-glucose was demonstrated unequivocally in separate control experiments (see the Experimental section). Deoxy and deoxyfluoro analogues of D-glucose carrying substitutions at C-3, C-4 or C-6 were not detectably turned over by trehalose phosphorylase. Note that 1,5-anhydroglucitol, which lacks the reacting 1-OH, is also not a substrate of the enzyme [19].

Judging by  $k_{\text{cat}}/K_m$ , the alternative glucosyl acceptors were poorer substrates than D-glucose. The results in Table 1 reveal an important role of the C-2(R) hydroxy group of D-glucose for the catalytic competence of trehalose phosphorylase. Interestingly, 2-amino-2-deoxy-D-glucose was found not to be a substrate of trehalose phosphorylase. It was a competitive inhibitor against D-glucose with a  $K_i$  of  $46 \pm 7$  mM (mean  $\pm$  S.D.), which, however, indicates binding to the enzyme–Glc1P complex. By using the expression  $\Delta\Delta G = -RT \ln[(k_{\text{cat}}/K_m)^{2\text{-deoxy-Glc}} / (k_{\text{cat}}/K_m)^{\text{Glc}}]$ , a binding energy of approx. 9 kJ/mol was calculated to be used by the enzyme for the stabilization of the transition state of glucosyl transfer and probably derived from hydrogen-bonding interactions with the 2-OH. The closely similar catalytic efficiencies for the enzymic reactions with 2-deoxy-D-glucose and 2-deoxy-2-fluoro-D-glucose suggest that this hydroxy group might function

as a donor of a hydrogen for bonding in the transition state of the enzymic reaction (see [28] for a general discussion).

The observed effects on  $k_{\text{cat}}/K_m$  for the reaction of trehalose phosphorylase with the D-glucose analogues were distributed between a 25-fold decrease in  $k_{\text{cat}}$  and an 18-fold increase in  $K_m$  (Table 1). The significant variation of  $k_{\text{cat}}$  in response to relatively small, structurally conservative modifications [28] of the glucosyl acceptor implies an important contribution of reaction chemistry to rate limitation of the enzymic synthesis of  $\alpha$ -D-glucosides.

### Inhibition of the trehalose-phosphorylase-catalysed reaction by vanadate

Vanadate was found to inhibit strongly the forward and reverse reaction catalysed by trehalose phosphorylase. Other anions such as molybdate, sulphate, thiophosphate, phosphite and nitrate were between 20000-fold and 100000-fold poorer inhibitors of the enzyme. Vanadate acted as a competitive inhibitor against P<sub>i</sub> when  $\alpha,\alpha$ -trehalose was present at a constant saturating concentration, and against Glc1P when D-glucose was present at a constant saturating concentration (results not shown).  $K_i$  values for vanadate were obtained from non-linear fits of the data to eqn (2) and are summarized in the rightmost column in Table 1. The results suggest that vanadate ( $K_i \approx 1 \mu\text{M}$ ) binds approx. 26000-fold and 2400-fold more tightly than P<sub>i</sub> ( $K_i = 26$  mM [19]) and Glc1P ( $K_i = 2.4$  mM [19]) respectively.

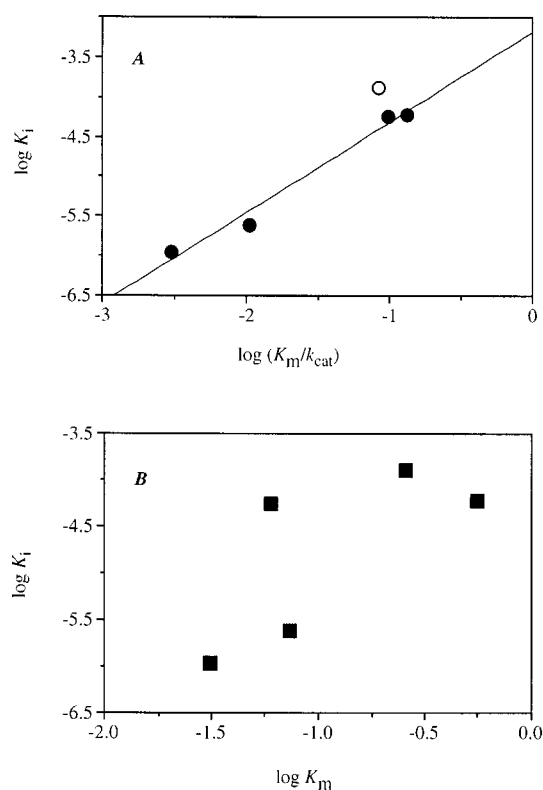
When D-glucose was replaced by one of the substrate analogues in Table 1 and the inhibition by vanadate against Glc1P was evaluated again, the  $K_i$  values thus obtained were much greater than that observed in the presence of D-glucose; they are summarized in Table 1. The experimental  $K_i$  value,  $K_i$  (obs), was clearly dependent on the respective glucosyl acceptor used in the experiment and therefore cannot represent the dissociation constant of vanadate from the binary complex with trehalose phosphorylase. This unexpected result implies an adduct of vanadate and the glucosyl acceptor to be the actual inhibitor of trehalose phosphorylase.

It is well known that vanadate reacts rapidly with alcohols in aqueous solution to give esters [26,27,29]. Therefore the formation of the inhibitory adduct with vanadate could in principle occur both at the active site of trehalose phosphorylase and in solution. Our results do not allow us to distinguish clearly between these two possibilities. However, a single piece of evidence supports the preferred model, namely binding of the glucosyl acceptor to the enzyme–vanadate complex and the formation of a non-covalent adduct but not an ester. It seems reasonable to assume that, irrespective of the mechanism of its formation, any inhibitory covalent adduct with vanadate should

**Table 1** Kinetic parameters of trehalose phosphorylase for reactions with substrates and substrate analogues and apparent binding constants for the inhibitor vanadate

$K_i^{\text{vanadate}}$  was determined from non-linear fits to eqn (2) of initial-velocity data recorded in the presence of varied concentrations of Glc1P and P<sub>i</sub> and constant concentrations of D-glucose or an analogue of D-glucose (100 mM), and  $\alpha,\alpha$ -trehalose (400 mM) respectively. For synthesis measurements, Glc1P concentration was held constant at 27 mM; for phosphorylase measurements, P<sub>i</sub> concentration was held constant at 50 mM. Abbreviation: n.d., not determined. Results are means  $\pm$  S.D.

Reaction type	Varied substrate	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ (M <sup>-1</sup> ·s <sup>-1</sup> )	$K_i^{\text{vanadate}}$ ( $\mu\text{M}$ )
Synthesis	D-Glc	10.3 $\pm$ 0.3	31 $\pm$ 4	332	1.1 $\pm$ 0.1
	2-Deoxy-D-glucose	0.61 $\pm$ 0.20	60 $\pm$ 9	10.2	56 $\pm$ 13
	2-Deoxy-2-fluoro-D-glucose	3.1 $\pm$ 0.2	257 $\pm$ 26	12.1	128 $\pm$ 36
	D-Mannose	0.42 $\pm$ 0.02	55 $\pm$ 10	7.6	n.d.
	2-Keto-D-glucose	4.2 $\pm$ 0.4	553 $\pm$ 82	7.6	59 $\pm$ 17
	5-Thio-D-glucose	7.0 $\pm$ 0.3	74 $\pm$ 9	95	2.4 $\pm$ 0.2
Phosphorolysis	$\alpha,\alpha$ -Trehalose	14.1 $\pm$ 0.2	87 $\pm$ 4	162	0.75 $\pm$ 0.10



**Figure 1** Linear free-energy relationship between kinetic parameters for the inhibitor vanadate ( $K_i$ ) and the catalytic efficiency of enzymic glucosyl transfer from phosphate ( $k_{cat}/K_m$ ) (A) and the apparent binding constant ( $K_m$ ) of the glucosyl acceptor (B)

The data are taken from Table 1. The line in (A) represents the best linear fit of the data, excluding the value shown by an open circle (reactions with 2-deoxy-2-fluoro-D-glucose).

involve the 1-OH of the glucosyl acceptor. Now, inhibition by vanadate was seen in the direction of phosphorolysis of  $\alpha,\alpha$ -trehalose (see Table 1); the disaccharide does not contain a free 1-OH. The formation of a complex between the enzyme, vanadate and D-glucose could contribute to the observed inhibition when  $\alpha,\alpha$ -trehalose is saturating, thus leaving some uncertainty. However, a ternary inhibitor complex of enzyme, vanadate and  $\alpha,\alpha$ -trehalose was clearly demonstrated in double-inhibition experiments in the absence of phosphate (see below).

Assuming rapid-equilibrium ordered binding of the inhibitor components,  $K_i$  (obs) is given by the expression  $K_i/(1 + [B]/K'_B)$  where  $K_i$  is the true dissociation constant of the enzyme–vanadate complex, [B] is the concentration of D-glucose or a derivative thereof, and  $K'_B$  is the dissociation constant of B from the ternary inhibitor complex. It is therefore the value of  $K'_B$  that changes in response to relatively small structural modifications of the glucosyl acceptor.

Figure 1(A) shows a double-logarithmic plot of the reciprocal catalytic efficiencies for the trehalose-phosphorylase-catalysed glucosyl transfer to D-glucose and derivatives thereof, and the corresponding  $K_i$  for the inhibition by vanadate. The plot reveals a reasonably good linear correlation between  $\log K_i$  and  $\log(K_m/k_{cat})$ . The coefficient of determination ( $r^2$ ) was 0.979, and a slope of 1.14 was obtained. Structural alterations of the glucosyl acceptor therefore produced parallel effects of a closely similar magnitude on the enzymic rate enhancement and the affinity of the inhibitor. This is considered to be a rigorous

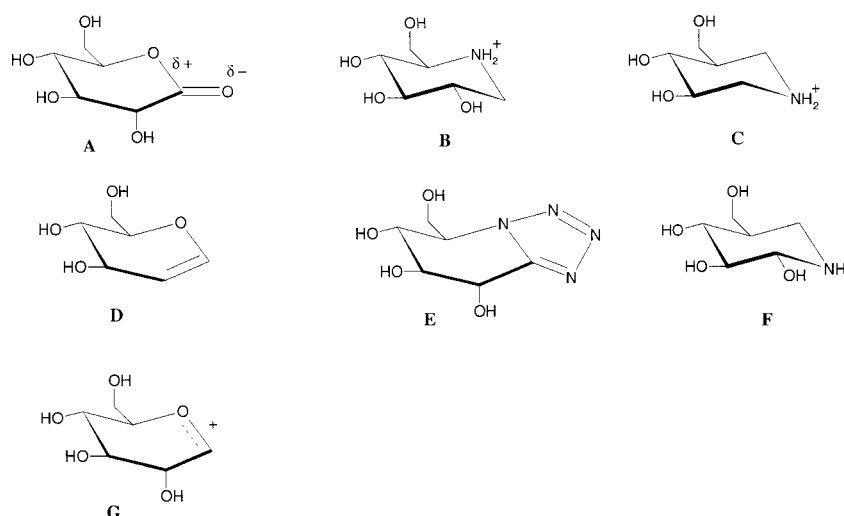
criterion of transition-state analogy [30]. Note, however, that the slope value does not represent a measure of how well the inhibitor approximates the transition state. The quantitative relationship embodied in the expression  $\log K_i \propto \log(K_m/k_{cat})$  usually serves to stress the difference between a true transition-state analogue and a two-substrate analogue that mimics the combined binding of the substrates in the ground-state form. However, the possibility of a uniform binding case in which binding is expressed both at the ground state and transition state of the reaction must be considered. An important requirement for the above analysis to be valid is that the chemical step of the enzymic reaction is rate-limiting across the series of substrates chosen. It seems to be fulfilled for the trehalose-phosphorylase-catalysed glucosyl transfer from phosphate, as follows. Because ternary-complex interconversion for the reverse reaction of trehalose phosphorylase with the natural substrates has been shown to be partly rate-limiting [19], it is reasonable to assume rate-limiting chemistry for the enzymic reactions with alternative glucosyl acceptors that are poorer substrates than D-glucose. Furthermore, if chemistry is mainly rate-limiting for the enzymic reaction overall, a decrease in  $k_{cat}/K_m$  observed across a series of homologous substrates is expected to produce an effect of the same magnitude in the corresponding  $k_{cat}$ . This trend is generally observed in Table 1. Among other enzyme systems, the relationship of  $\log K_i$  against  $\log(K_m/k_{cat})$  has been used to distinguish true transition-state inhibitors of glycosidases [31] and GTs [32] from fortuitously tightly binding inhibitors.

A plot of  $\log K_m$  against  $\log K_i$  showed no useful linear correlation of the two parameters for trehalose phosphorylase (Figure 1B), apparently suggesting the absence of ground-state mimicry provided by the inhibitor. However, caution must be exercised generally in attempting to interpret a relationship between  $K_m$  and  $K_i$ . The degree to which  $K_m$  truly reflects the 'binding' of the glucosyl acceptor to the enzyme–Glc1P complex might be quite small for trehalose phosphorylase: the  $K_m$  for D-glucose is 40 mM but the dissociation constant of the ternary complex with D-glucose is only 1.4 mM [19].

A basic question, unanswered by the present results, is why the adduct of vanadate and D-glucose displays partial transition-state analogy instead of simply mimicking the ground-state binding of phosphate and D-glucose. Inorganic vanadate resembles phosphate in many respects; the  $pK_a$  values of all three ionizations of vanadic acid are similar to those of phosphoric acid [33]. However, the V–O bonds are reported to be approx. 0.15 Å (1 Å  $\equiv$  0.1 nm) or 10% longer than the corresponding P–O bonds [33], which could be a relevant factor. It has also been suggested that an increase in the V–O bond distance might not be as demanding, energetically, as in the P–O bond [33]. We therefore envisage that the interaction between vanadate and the 1-OH of D-glucose involves a stretched V–O bond that enables the formation of a hydrogen bond between the two molecules. The polarity of this hydrogen bond and the active ionic form of vanadate are not revealed by our data. However, taking into consideration that  $H_2PO_4^-$  is the substrate of trehalose phosphorylase in the phosphorolysis direction [19], we expect that  $H_2VO_4^-$  forms the adduct with  $\alpha,\alpha$ -trehalose by donating a hydrogen for bonding to the glycosidic oxygen. Therefore an interaction of the form RO–H ... O–VHO<sub>3</sub><sup>2-</sup> involving the 1-OH as hydrogen bond donor would seem likely but is not proved.

#### Inhibition of trehalose phosphorylase by partial D-glucosyl-cation analogues

The common use of D-glucono- $\delta$ -lactone and azasugars such as isofagomine or 1-deoxynojirimycin as reversible inhibitors of



**Figure 2** Structures of inhibitors used in the experimental study, and their partial mimicry of the oxocarbenium-ion-like transition state of GHs and GTs

(A) D-Glucono- $\delta$ -lactone; (B) 1-deoxynojirimycin; (C) isofagomine; (D) D-glucal; (E) nojirimycin tetrazole; (F) noeujirimycin; (G) glucosyl cation.

**Table 2** Dissociation constants of complexes between trehalose phosphorylase and some typical inhibitors of glucosidases and glucosyltransferases

For E-P<sub>i</sub> measurements the  $\alpha,\alpha$ -trehalose concentration was varied and P<sub>i</sub> was held constant at 40 mM; for E-Glc1P measurements the D-glucose concentration was varied and Glc1P was held constant at 40 mM. With  $\alpha$ -methyl-D-glucoside the maximum concentration of inhibitor was 200 mM. Abbreviations: TS, transition state; GS, ground state (possible modes of binding); uc, uncompetitive inhibitor. Results are means  $\pm$  S.D.

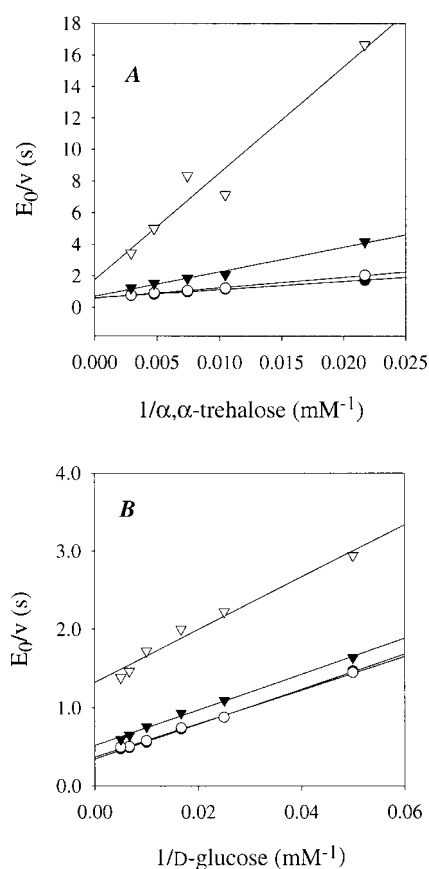
Inhibitor	Enzyme form ...	K <sub>i</sub> (mM)	
		E-P <sub>i</sub> (GS, TS)	E-Glc1P (GS)
D-Glucal		0.31 $\pm$ 0.03	0.32 $\pm$ 0.04
D-Glucono- $\delta$ -lactone		0.24 $\pm$ 0.02	95 $\pm$ 22
1-Deoxynojirimycin		1.2 $\pm$ 1.1	6.8 $\pm$ 1.1
Isofagomine		0.056 $\pm$ 0.006	0.8 (uc)
$\alpha$ -Methyl D-glucoside		No inhibition	No inhibition

glycosidases [1,2,34–36] and GTs [1,2,37] reflects the idea that the reactions catalysed by members of both enzyme classes involve transition states with substantial oxocarbenium ion character. The inability of trehalose phosphorylase to use 2-deoxy-2-fluoro- $\alpha$ -D-glucose 1-phosphate in place of Glc1P (C. Eis and B. Nidetzky, unpublished work) is in good agreement with this notion because a major effect of the fluorine at C-2 would be the inductive destabilization of a glucosyl-cation-like transition state. Compounds that mimic the sp<sup>2</sup>-hybridized anomeric centre and the consequential partly planar conformation of the glucopyranosyl ring, and the build-up and distribution of positive charge of the oxocarbenium ion in the transition state should therefore be good inhibitors of the hydrolases and the transferases. The partial analogy with the glucosyl cation of four inhibitors used in this study is emphasized in Figures 2(A)–2(D). Table 2 summarizes the dissociation constants of these inhibitors from the binary trehalose phosphorylase complexes.

D-Glucal and 1-deoxynojirimycin inhibited competitively against D-glucose. (Note that D-glucal is not a substrate of trehalose phosphorylase [19].) Both inhibitors are therefore

capable of mimicking the ground-state binding of D-glucose with the enzyme-Glc1P complex. The dissociation constants of D-glucal and 1-deoxynojirimycin from the enzyme-Glc1P and enzyme-P<sub>i</sub> complexes were similar. D-Glucono- $\delta$ -lactone showed a 400-fold preference for binding with enzyme-P<sub>i</sub> over binding with enzyme-Glc1P, probably indicating a poor affinity of the lactone for sugar-binding subsite +1 of trehalose phosphorylase. Isofagomine bound tightly with the enzyme-P<sub>i</sub> complex and inhibited competitively against  $\alpha,\alpha$ -trehalose (Figure 3A). The much weaker, uncompetitive inhibition observed when enzyme-Glc1P was the prevailing enzyme form, with a small portion of enzyme-P<sub>i</sub> being present (Figure 3B), implies exclusive interactions of isofagomine with enzyme-P<sub>i</sub> and a large preference for binding at sugar-binding subsite -1. For comparison, the inhibition of trehalose phosphorylase by methyl- $\alpha$ -D-glucoside was measured. The glucoside did not show detectable binding with the enzyme-Glc1P and enzyme-phosphate complexes.

1-Deoxynojirimycin and isofagomine are believed to bind to enzyme active sites in the protonated form such that favourable interactions are possible between their positively charged endocyclic nitrogen atoms and negatively charged catalytic groups on the enzyme or an appropriately positioned functional group on the co-substrate. However, they differ in the position on the pyranose ring in which they apparently generate the positive charge (see Figure 2). This difference is generally used to explain the rather selective profile of each compound with regard to the inhibition of  $\alpha$ - and  $\beta$ -retaining enzymes: 1-deoxynojirimycin is usually a powerful inhibitor of  $\alpha$ -glucosidases but is a weaker inhibitor of  $\beta$ -glucosidases. Isofagomine is reported to show a complementary inhibitory profile [2,4,34,38]. Considering this and assuming mechanistic analogies between  $\alpha$ -retaining GH and GT reactions [1,8,37], it was unexpected that trehalose phosphorylase was strongly inhibited by isofagomine, whereas 1-deoxynojirimycin displayed weak, if any, binding with sugar-binding subsite -1. The observed inhibitory profile for trehalose phosphorylase suggests that the anomeric carbon arguably receives a greater share of positive charge than the ring oxygen on development of the oxocarbenium ion in the transition state of enzymic glucosyl transfer. This charge distribution on the glucosyl cation would be strongly favoured by interactions taking



**Figure 3** Reversible inhibition of trehalose phosphorylase by isofagomine in forward (A) and reverse (B) enzymic reactions

Initial velocities were recorded in the presence of 40 mM phosphate (A) and 40 mM Glc1P (B). The isofagomine concentrations (in mM) used were: (A) ●, 0; ○, 0.013; ▼, 0.13; ▽, 1.31; (B) ●, 0; ○, 0.030; ▼, 0.26; ▽, 2.60.

place from the front ( $\alpha$ ) side of the sugar ring rather than from the back ( $\beta$ ) side [4]. On the front side of the glucosyl cation, the phosphate molecule seems to be appropriately positioned to provide these interactions (with the 2-OH, for example [4]).

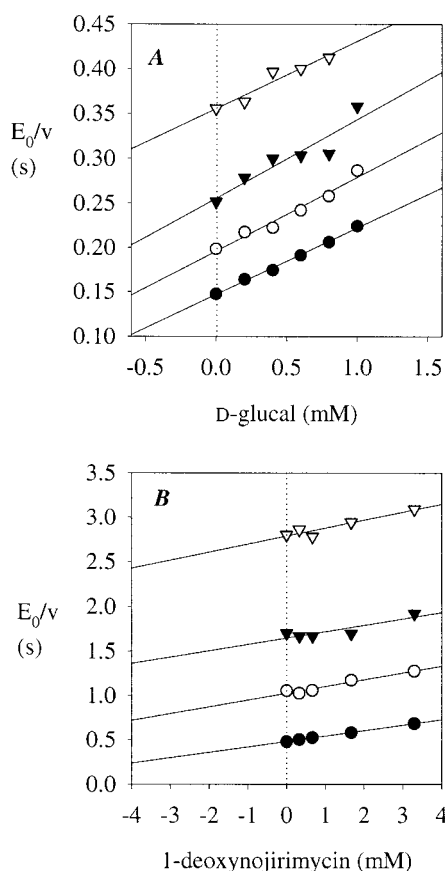
Previous studies of the interactions of  $\alpha$ 1,3-galactosyltransferase and glycogen phosphorylase with glycosyl-cation analogues are relevant for the present work with trehalose phosphorylase. The comparison with both GTs is particularly interesting because the chemical mechanisms used by  $\alpha$ -retaining  $\alpha$ 1,3-galactosyltransferase [13] and glycogen phosphorylase [16] seem to differ fundamentally with regard to the participation of an enzyme nucleophile in catalysis. Like trehalose phosphorylase,  $\alpha$ 1,3-galactosyltransferase was inhibited strongly by an azasugar of the isofagomine class, whereas the corresponding deoxynojirimycin compound did not show detectable binding [39]. Kinetic evidence for binding of the isofagomine-type inhibitor to the enzyme-UDP complex was obtained [39]. The recently solved X-ray structure of the enzyme [13] suggests that in spite of the presence of the nucleophile at the  $\beta$ -face of the sugar, charge-charge interactions between N-1 of the inhibitor and the oxygen of the  $\beta$ -phosphate group of UDP might contribute to the stability of the ternary complex.

The binding of glucosyl cation analogues only with the enzyme-phosphate complex of trehalose phosphorylase might be considered an extreme case of the synergistic binding of inhibitors

and phosphate observed with glycogen phosphorylase [16]. The inhibitory profiles of 1-deoxynojirimycin and D-glucono- $\delta$ -lactone with regard to glycogen phosphorylase (summarized in [40]) and trehalose phosphorylase are also quite similar. The interaction of glycogen phosphorylase with nojirimycin tetrazole (Figure 2E) has been studied by X-ray diffraction and kinetic analysis [40]. Tight binding of the inhibitor required phosphate ( $K_i = 53 \mu\text{M}$ ), and contacts between N-1 and O-2 of the inhibitor and the oxygen atom of phosphate in the ternary complex were shown to determine ligand synergism. The combination of  $\text{sp}^2$  hybridization at the anomeric carbon, the polar nature of the tetrazole ring and phosphate was suggested to provide essential elements of transition-state analogy and thus high-affinity binding with glycogen phosphorylase [40]. For trehalose phosphorylase, the electrostatic component of the transition state of glucosyl transfer might be partly realized when isofagomine binds to the enzyme-phosphate complex. However, contributions to binding that depend on half-chair geometry of the glucosyl cation as well as the 2-OH are lacking in the ternary complex with isofagomine. The relatively similar specificities of glycosyl-cation analogues for binding reversibly to trehalose phosphorylase,  $\alpha$ 1,3-galactosyltransferase and glycogen phosphorylase shows that caution must be exercised in trying to deduce mechanistic information from a comparative analysis of the inhibition profiles. It is interesting in this context that noeuromycin (Figure 2F), which (unlike isofagomine) contains a hydroxy group adjacent to the ring nitrogen at the position corresponding to the 2-OH of the sugar bound at the catalytic subsite, was recently found to be a tight but stereochemically non-selective inhibitor of retaining glucosidases [41]. This suggests that the endocyclic nitrogen of noeuromycin is capable of interacting efficiently with a functional, probably catalytic group of both the  $\alpha$ - and  $\beta$ -retaining enzyme. The X-ray structure of  $\beta$ -N-acetylhexosaminidase complexed with GalNAc-isofagomine revealed a strong hydrogen bond between a proton of the 'anomeric' nitrogen of the inhibitor and the carboxylate group of the acid-base catalyst, suggesting that tight binding of the azasugar does not necessarily require charge-charge interactions with the enzyme [42]. It is noteworthy that  $\beta$ -N-acetylhexosaminidase is a retaining hydrolase that lacks a catalytic nucleophile and uses assistance by the carbonyl oxygen of the 2-acetamido group of the substrate for catalysis. Strong inhibition by an azasugar of the isofagomine class was therefore unexpected for this enzyme and stresses the need to consider different inhibitor-binding modes in interpreting observed azasugar specificities.

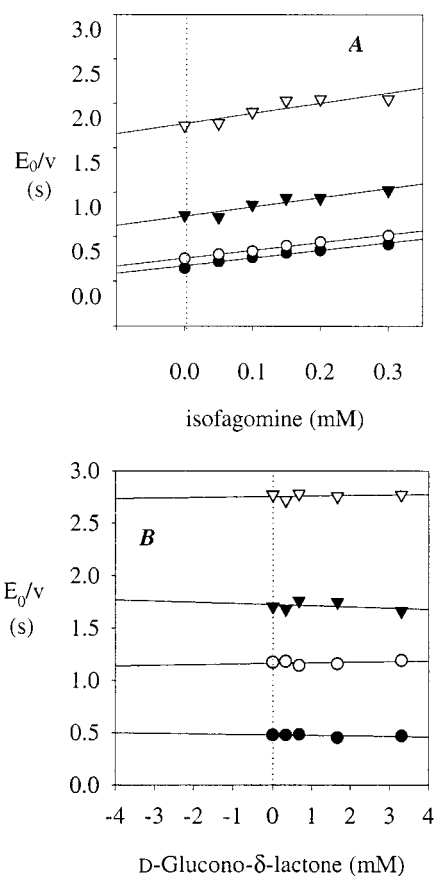
### Double-inhibition studies

Kinetic studies with a single inhibitor could not determine unambiguously whether binding of this inhibitor occurred to only one sugar-binding subsite at the active site of the enzyme or to both subsites simultaneously (see Table 2). The double-inhibition studies were performed to resolve these outstanding issues and determine the relative affinities of the above described inhibitors to each of the two subsites. Yonetani-Theorell plots for the inhibition by different inhibitor pairs of the forward and reverse reactions catalysed by trehalose phosphorylase are shown in Figures 4-6. The results in Figures 4(A) and 4(B) reveal a parallel line pattern in each case, indicating that D-glucal (Figure 4A) and 1-deoxynojirimycin (Figure 4B) do not bind to the phosphorylase when vanadate is bound. The inhibitory effect of both inhibitors was as expected from the respective  $K_i$  values (Table 2). This therefore implies that no adduct is formed between either inhibitor and vanadate at the enzyme's active site, probably because both compounds lack the anomeric 1-OH of D-glucose.



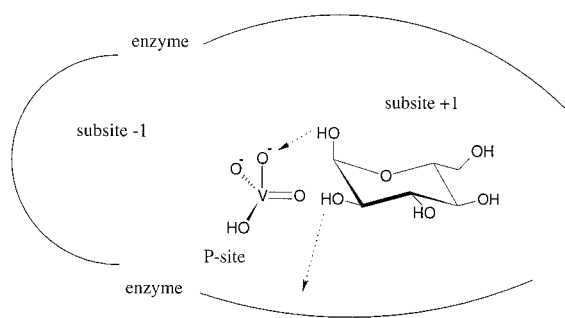
**Figure 4** Subsite binding of inhibitors of trehalose phosphorylase determined in double-inhibition experiments

Yonetani–Theorell plots for inhibition by D-glucal and orthovanadate (A) and by 1-deoxynojirimycin and orthovanadate (B). The orthovanadate concentrations (in  $\mu\text{M}$ ) used were: (A) ●, 0; ○, 2.2; ▼, 4.4; ▽, 8.8; (B) ●, 0; ○, 5; ▼, 10; ▽, 22. Reaction rates were measured in the direction of synthesis as the release of phosphate with 10 mM Glc1P and 250 mM D-glucose in 20 mM Mes buffer, pH 6.6.



**Figure 5** Subsite binding of inhibitors of trehalose phosphorylase determined in double-inhibition experiments

Yonetani–Theorell plots for inhibition by isofagomine and orthovanadate (A) and by D-glucono- $\delta$ -lactone and orthovanadate (B). The orthovanadate concentrations (in  $\mu\text{M}$ ) used were 0 (●), 2.2 (○), 4.4 (▼) and 8.8  $\mu\text{M}$  (▽). Reaction rates were measured in the direction of synthesis as the release of phosphate with 10 mM Glc1P and 250 mM D-glucose in 20 mM Mes buffer, pH 6.6.



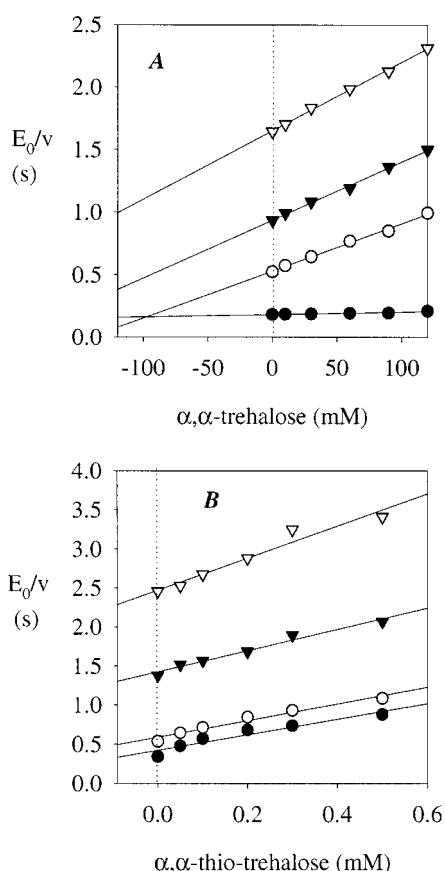
**Scheme 1** Model of the inhibition of trehalose phosphorylase by vanadate

The broken lines indicate hydrogen bonds; the arrows indicate the probable polarities of these bonds in the proposed ternary complex. The P-site is the site in which phosphate binds.

Because D-glucal was shown clearly to bind to subsite +1 of trehalose phosphorylase with high affinity [19], the D-glucose in the enzyme-bound vanadate/D-glucose adduct most probably occupies this subsite and thereby prevents any interaction of the enzyme with D-glucal (Scheme 1).

Figure 5 shows the inhibition of trehalose phosphorylase by isofagomine (Figure 5A) and D-glucono- $\delta$ -lactone (Figure 5B) in the absence and the presence of vanadate. The results reveal little, if any, binding of both inhibitors to subsite +1 under conditions in which no vanadate was added. This agrees well with Table 2. When vanadate was present, no additional inhibition was observed other than that brought about by vanadate alone. The results therefore rule out the formation of an adduct between vanadate and D-glucono- $\delta$ -lactone, or between vanadate and isofagomine. Considering the proposed binding mode for vanadate and D-glucose in Scheme 1 and the ordered reaction mechanism of trehalose phosphorylase, which requires occupancy of the phosphate-binding site before sugar binding to subsite -1 can occur, it becomes quite clear that the binding of isofagomine and vanadate, and similarly D-glucono- $\delta$ -lactone and vanadate, must be mutually exclusive. Only if the enzyme-phosphate complex is present in appreciable concentrations will the two inhibitors show detectable binding and then to sugar-binding subsite -1.

Figure 6 shows the inhibition of trehalose phosphorylase by the inhibitor pairs  $\alpha,\alpha$ -trehalose/vanadate (Figure 6A) and  $\alpha,\alpha$ -thiotrehalose/vanadate (Figure 6B). In the absence of vanadate,  $\alpha,\alpha$ -trehalose did not bind to the free enzyme and showed no



**Figure 6** Subsite binding of inhibitors of trehalose phosphorylase determined in double-inhibition experiments

Yonetani–Theorell plots for inhibition by  $\alpha,\alpha$ -trehalose and orthovanadate (A), and by  $\alpha,\alpha$ -thio-trehalose and orthovanadate (B). The orthovanadate concentrations (in  $\mu\text{M}$ ) used were: (A) ●, 0; ○, 5.5; ▼, 11; ▽, 22; (B) ●, 0; ○, 1.7; ▼, 5; ▽, 10. Reaction rates were measured in the directions of synthesis with 10 mM Glc1P and 50 mM D-glucose (A), and phosphorolysis with 10 mM phosphate and 80 mM  $\alpha,\alpha$ -trehalose (B). In (A) the release of phosphate was determined; in (B) that of D-glucose was determined.

detectable inhibition of the reverse enzymic glucosyl transfer. (Because the concentration of Glc1P used in the experiment was approx. 6 times  $K^{\text{Glc1P}}$ , the binary complex and free trehalose phosphorylase were the prevailing enzyme forms in the steady state.) When vanadate was present, inhibition by  $\alpha,\alpha$ -trehalose was observed. The Yonetani–Theorell plot shows a parallel line pattern (Figure 6A), which apparently suggests that  $\alpha,\alpha$ -trehalose and vanadate were unable to bind to the enzyme's active site simultaneously. The conflict of this result is dispelled by considering that the formation of an enzyme-bound adduct of  $\alpha,\alpha$ -trehalose and vanadate must of necessity compete with the formation of the corresponding adduct of D-glucose and vanadate (see Scheme 1).

We used phosphite to determine whether the occupancy of the phosphate-binding site of trehalose phosphorylase by a phosphate analogue other than vanadate was sufficient to trigger the formation of an active-site scaffold capable of binding  $\alpha,\alpha$ -trehalose. Phosphite was shown to inhibit competitively against phosphate with a  $K_i$  of  $18 \pm 2$  mM. Therefore it binds to free trehalose phosphorylase about equally well as does the substrate phosphate. When phosphite and  $\alpha,\alpha$ -trehalose were used in double-inhibition studies of the reverse enzymic reaction, no

inhibition other than that contributed by phosphite alone was observed (results not shown). This indicates the inability of  $\alpha,\alpha$ -trehalose to bind with the enzyme–phosphite complex. The result could imply that phosphite is simply not a good enough analogue of phosphate and fails to induce a proper binding site of  $\alpha,\alpha$ -trehalose; an alternative, preferred explanation is that formation of the tight-binding adduct involves specific interactions between  $\alpha,\alpha$ -trehalose and the phosphate analogue at the enzyme's active site. These interactions might be available to vanadate but not to phosphite.

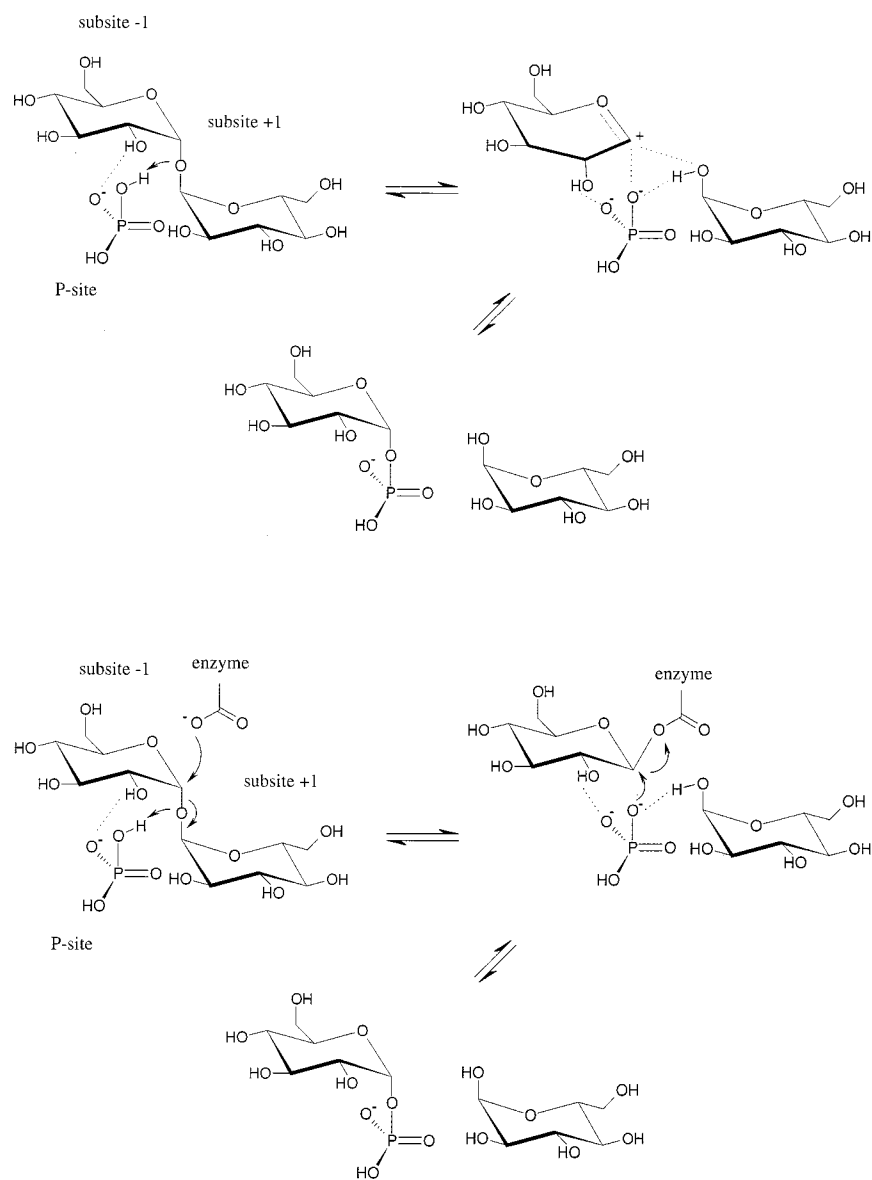
Results obtained with the inhibitor pair  $\alpha,\alpha$ -thio-trehalose and vanadate (Figure 6B) gave a parallel line pattern in the corresponding Yonetani–Theorell plot, as would be expected if the adduct of  $\alpha,\alpha$ -thio-trehalose and vanadate were the actual inhibitor. In this experiment, however, binding of  $\alpha,\alpha$ -thio-trehalose to the enzyme–phosphate complex would produce the same inhibition pattern. Because the concentration of  $P_i$  (10 mM) was 7 times  $K^{P_i}$ , whereas that of  $\alpha,\alpha$ -trehalose matched  $K^{\text{trehalose}}$ , a major proportion of the enzyme was present as the enzyme–phosphate complex in the steady state. The significant inhibition of trehalose phosphorylase by  $\alpha,\alpha$ -thio-trehalose when no vanadate was present provides good evidence for the formation of an abortive ground-state ternary complex, enzyme–phosphate– $\alpha,\alpha$ -thio-trehalose.

#### Implications for $\alpha$ -retaining glucosyl transfer catalysed by trehalose phosphorylase

The results obtained in this study can be consolidated in two mechanistic models that differ mainly with regard to the formation of a covalent intermediate and are summarized in Scheme 2. An internal return mechanism similar to that proposed for glycogen phosphorylase [43] is depicted in Scheme 2 (upper panel). The essential components of this mechanism are the interaction between the leaving group and the incoming nucleophile, and significant front-side stabilization of the oxocarbenium ion by electrostatic interactions with the substrate phosphate itself. The transition state in Scheme 2 (upper panel) would be expected to display a highly dissociative character. Internal return to yield the  $\alpha$ -retained product could occur from within the ion pair by the recombination of the anomeric carbon and the leaving group, which would be effectively  $\text{HO}_3\text{PO}^-\text{H} \dots \text{OR}$  and  $\text{HO}_3\text{PO}^{2-} \dots \text{H-OR}$  in the forward and reverse enzymic reactions respectively, on the oxygen of phosphate or the oxygen at C-1 of D-glucose. An attractive feature of the  $S_Ni$  mechanism is that a rationale is provided for (1) the transition state-like inhibition by  $\text{HO}_3\text{VO}^{2-} \dots \text{H-OR}$ , which would mimic the effective leaving group, and (2) the apparently inherent coupling of the bond-cleavage and bond-forming steps.

The glucosyltransferase mechanism involving a  $\beta$ -glucosyl enzyme intermediate is shown in Scheme 2 (lower panel). Precedent strongly favours this mechanism [1,44]. Consideration of the stability of the possible reaction intermediates clearly suggests a covalent adduct. Trehalose phosphorylase has a  $k_{\text{cat}}$  of approx.  $10 \text{ s}^{-1}$  for the phosphorolysis of  $\alpha,\alpha$ -trehalose. Because the reaction to give products is partly rate-limiting, this small  $k_{\text{cat}}$  requires non-chemical steps at the ternary-complex level to be slow, or a reaction intermediate that has a lifetime of the order of  $10^{-2}$  to  $10^{-3}$  s. The stability of an oxocarbenium ion in the presence of phosphate is expected to be on the borderline of real existence ( $10^{-12}$  to  $10^{-14}$  s) [1], calling into question whether an intermediate of the trehalose-phosphorylase-catalysed glucosyl transfer could be ionic. In Scheme 2 (lower panel), hydrogen-bonding interactions between phosphate and the 1-OH of D-





**Scheme 2** Possible mechanisms of glucosyl transfer catalysed by trehalose phosphorylase

Internal return (upper panel) and double inversion via covalent intermediate (lower panel). Non-covalent bonding is indicated by broken lines. The hydrogen bond between the 2-OH of the glucosyl moiety and the phosphate oxygen is tentative [16].

glucose could take place in the deglycosylation step, which arguably explains the observed inhibition of trehalose phosphorylase by an  $\text{HO}_3\text{PO}^{2-} \dots \text{H-OR}$  adduct.

An attractive component of both mechanisms in Scheme 2 is that  $\text{HO}_3\text{PO}^{2-}$  could fulfil the role of the proton donor of the reaction, providing catalytic assistance to glycosidic bond cleavage in  $\alpha,\alpha$ -trehalose. According to the principle of microscopic reversibility, the  $\text{HO}_3\text{PO}^{2-}$  group would function as the catalytic base for the nucleophilic addition of D-glucose in reverse reaction. Interestingly, a similar role for the substrate phosphate was suggested more than 20 years ago for the enzymic glucosyl transfer catalysed by  $\alpha$ -glucan (glycogen) phosphorylase (reviewed in [43]); this perceptive proposal has received support from the recently solved X-ray structure of the enzyme in the presence of substrate analogue [16].

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