Catalytic mechanism of α -retaining glucosyl transfer by *Corynebacterium callunae* starch phosphorylase: the role of histidine-334 examined through kinetic characterization of site-directed mutants

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Purified site-directed mutants of *Corynebacterium callunae* starch phosphorylase in which His-334 was replaced by an alanine, glutamine or asparagine residue were characterized by steady-state kinetic analysis of enzymic glycosyl transfer to and from phosphate and studies of ligand binding to the active site. Compared with wild-type, the catalytic efficiencies for phosphorolysis of starch at 30 °C and pH 7.0 decreased approx. 150- and 50-fold in H334Q (His³³⁴ → Gln) and H334N mutants, and that of H334A was unchanged. In the direction of α -glucan synthesis, selectivity for the reaction with G1P (α -D-glucose 1-phosphate) compared with the selectivity for reaction with α -D-xylose 1-phosphate decreased from a wild-type value of ~20000 to 2600 and 100 in H334N and H334Q respectively. Binding of G1P to the free enzyme was weakened between 10-fold (H334N, H334Q) and 50-fold (H334A) in the mutants, whereas binding to the complex of enzyme and α -glucan was not affected. Quenching of fluorescence of the pyridoxal 5'-phosphate cofactor was used to examine interactions of the inhibitor GL (D-gluconic acid 1,5-lactone)

with wild-type and mutant enzymes in transient and steady-state experiments. GL binding to the free enzyme and the enzyme-phosphate complex occurred in a single step. The 50-fold higher constant (K_d) for GL dissociation from H334Q bound to phosphate resulted from an increased off-rate for the ligand in the mutant, compared with wild-type. A log-log correlation of catalytic-centre activity for phosphorolysis of starch with a reciprocal K_d value established a linear free-energy relationship (slope = 1.19 ± 0.07 ; $r^2 = 0.991$) across the series of wild-type and mutant enzymes. It reveals that GL in combination with phosphate has properties of a transition state analogue and that the His-334 side chain has a role in selectively stabilizing the transition state of the reaction.

Key words: *Corynebacterium callunae*, glycogen phosphorylase, glycosyltransferase, maltodextrin, retaining mechanism, site-directed mutagenesis.

INTRODUCTION

Glycogen phosphorylases (GPs; EC 2.4.1.1) constitute a large family of GTs (glycosyltransferases) and occur in all three domains of life. In the systematic sequence-based classification of GTs, the GPs are found in the family GT-35, belong to clan IV and have a GT-B fold [1,2]. They catalyse the reversible phosphorolysis of α -glucan substrates such as maltodextrins, starch and glycogen, as shown in the following equation:

where N is the degree of polymerization of the substrate. In the phosphorolysis direction, GPs remove a glucosyl residue from the non-reducing end of the donor α -glucan which is positioned, through non-covalent interactions, at sugar-binding subsites -1 to $+4$ [3] and transfer it to the acceptor phosphate [4–6]. Bond cleavage takes place between subsites -1 and $+1$. Substrate binding to GPs is random, and a ternary complex must be formed before the first product is released [7,8]. Interconversion of central complexes is supposed to be rate-limiting at the steady state and occurs through transition state(s) with substantial oxocarbenium ion character [7,9]. The chemical conversion proceeds with retention of configuration at the anomeric carbon, which undergoes nucleophilic substitution in an axial-to-axial reaction [10]. The underlying catalytic mechanism of GPs is not well understood

[10,11]. Precedent with retaining GHs (glycosyl hydrolases) would strongly favour a two-step mechanism involving two configurationally invertive steps [9,12]: (i) cleavage of the carbon-oxygen bond between glucosyl residues bound in subsites -1 and $+1$ and formation of a covalent β -glucosyl-enzyme intermediate and (ii) reaction of the intermediate with phosphate to yield G1P (α -D-glucose 1-phosphate). At present, there is no evidence, structural, kinetic or otherwise, of the formation of a covalent glucosyl enzyme intermediate in the catalytic mechanism of GPs.

Crystal structures of *EcMalP* (*Escherichia coli* maltodextrin phosphorylase) ternary complexes have revealed the absence of a protein side chain appropriately placed to be a candidate nucleophile for promoting the covalent intermediate through a β -side attack of the reactive anomeric carbon [10,11]. The main chain carbonyl group of His-345 is directed towards O-5 and C-1 of the sugar in subsite -1 [10]. With a distance of 3.2 Å (1 Å = 0.1 nm) to both atoms, it assumes the approximate position of the canonical nucleophile in GHs, i.e. the carboxylate group of aspartic acid or glutamic acid [9,12,13]. It could provide a significant electrostatic stabilization of the oxocarbenium ion-like transition state through the partial negative charge on oxygen (Figure 1) and even function in covalent bonding (see [9] for a review). The side chain of His-345 participates in a hydrogen bond with the C-6 hydroxy group of the sugar in subsite -1 [10,11]. This interaction may have a 2-fold role in enzymic catalysis: (i) it contributes to a

Abbreviations used: CcStP, *Corynebacterium callunae* starch phosphorylase; EcMalP, *Escherichia coli* maltodextrin phosphorylase; G1P, α -D-glucose 1-phosphate; GH, glycosyl hydrolase; GL, D-gluconic acid 1,5-lactone; GP, glycogen phosphorylase; GT, glycosyltransferase; LFER, linear free-energy relationship; NJT, nojirimycin tetrazole; PLP, pyridoxal 5'-phosphate; X1P, α -D-xylose 1-phosphate.

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Figure 1 Proposed oxocarbenium ion-like transition state of glucosyl transfer by α -glucan phosphorylase and its stabilization by a conserved histidine

His-345 of *EcMalP* is equivalent to His-334 in *CcStP*, shown in parentheses. The reaction takes place from an obligatory ternary complex. In addition to providing electrostatic stabilization of the transition state, the amide carbonyl group of the histidine could function as a nucleophile in the catalytic mechanism [9].

positioning of the substrate in subsite -1 and (ii) directs a lone pair of electrons on the C-6 OH to the C-1-O-5 bond, thereby promoting and stabilizing the oxocarbenium ion. His-345 is universally conserved in members of the family GT-35 that have confirmed GP activity, emphasizing its importance in the catalytic mechanism of α -retaining glucosyl transfer.

In a thorough investigation, Schinzel and co-workers [14–17] have used site-directed mutagenesis to probe the role of individual side chains in different parts of the *EcMalP* active site. These authors replaced His-345 by an alanine residue but were not able to isolate and characterize the mutant [14], probably because it did not fold properly during recombinant protein production. Structure-based sequence comparisons unequivocally identify His-334 of *CcStP* (*Corynebacterium callunae* starch phosphorylase) to be the functional equivalent of His-345 of *EcMalP* [18]. We have substituted His-334 with alanine, glutamine and asparagine and report a characterization of purified mutants of *CcStP* using steady-state kinetic analysis of enzymic glucosyl transfer to and from phosphate, and inhibitor-binding studies both at equilibrium and in the kinetic transient. The kinetic consequences of the site-specific replacements of His-334 suggest a role for the original side chain in providing a stabilization energy that is specific to the transition state of the reaction and may be partly promoted through the C-6 OH of the sugar in subsite -1.

Numbering of amino acids in *CcStP* starts with 1 for the authentic N-terminal serine residue. The initiator methionine is processed off in the recombinant enzyme produced in *E. coli*.

EXPERIMENTAL

Materials

Materials for mutagenesis, protein purification and enzymic assays were described elsewhere [18,19]. All other chemicals were of the highest purity available and obtained from Sigma.

Mutagenesis

Mutations were generated with the Quik Change site-directed mutagenesis kit (Stratagene) according to the recommended standard method. The following oligonucleotide primers (and the reverse complementary primers) were used: 5'-CGTACACCA-

CGCCACCGTGCTCACC GAAG-3' (H334A, His³³⁴ → Ala), 5'-GCGTACACCAACCAACACCGTGCTCACC GAAG-3' (H334N) and 5'-GCGTACACCAACCAAAACCGTGCTCACC GAAG-3' (H334Q). Mutated codons are indicated in boldface. The plasmid pQE 70-StP [18] containing the gene for wild-type *CcStP* was used as the template in all cases. The mutagenized plasmids were transformed into *E. coli* JM109 cells. The mutant sequences were subjected to dideoxy sequencing at the VBC-Genomics Sequence Service Facility of the University of Vienna to confirm that the desired mutations had been introduced and no other mutations had occurred because of DNA polymerase errors.

Gene expression, purification and structural characterization of the recombinant enzyme

Recombinant wild-type *CcStP* was produced and purified according to procedures reported recently [18]. Expression of mutagenized *CcStP* genes in *E. coli* was performed as described previously [18]. The cells were suspended in 50 mM potassium phosphate buffer (pH 7.0), disrupted by repeated passage through a French press, followed by ultracentrifugation at 80 000 *g* for 30 min at 4 °C to remove the cell debris. Cell extract was brought to 25 % saturated ammonium sulphate and precipitated proteins were separated by ultracentrifugation. The clear supernatant was applied to a phenyl-Sepharose Fast Flow column (Amersham Biosciences), equilibrated with 50 mM potassium phosphate buffer (pH 7.0), containing 25 % saturated ammonium sulphate. The bound protein was eluted with 50 mM potassium phosphate buffer (pH 7.0). Fractions containing enzyme activity were pooled and concentrated using Vivaspin ultrafiltration concentrator tubes with a molecular mass cut-off of 10 kDa (Vivascience, Lincoln, U.K.). The protein solution was brought to 300 mM potassium phosphate (pH 7.0) and incubated at 60 °C for 30 min. The precipitated protein was removed by centrifugation at 16 000 *g* for 10 min at 4 °C. The supernatant was loaded on to a Fractogel EMD-DEAE column (Merck) equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The bound protein was eluted with a linear gradient from 0 to 1 M sodium chloride in the same buffer. After concentration of the pooled fractions, the protein was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) and again concentrated to approx. 10 mg/ml in 50 mM potassium phosphate buffer (pH 7.0). The mutants were stored at 4 °C. Protein concentration was determined using the Bio-Rad dye-binding method with BSA as the standard. Purification of the mutants was monitored by SDS gel electrophoresis and measurement of specific enzyme activity.

Before further use, the enzyme storage solution was gel-filtered using NAP 5 and NAP 10 columns (Amersham Biosciences), equilibrated with 50 mM triethanolamine buffer (pH 7.0). It has been proved that the concentration of phosphate was reduced to <0.2 mM. The content of PLP (pyridoxal 5'-phosphate) in wild-type and mutant enzymes was determined as described by Wada and Snell [20]. CD spectroscopic measurements were carried out as described recently [21] using enzyme solutions (0.3 mg/ml) in 50 mM potassium phosphate buffer (pH 7.0).

Enzymic assays

Enzymic activity was assayed in the direction of phosphorolysis in a coupled assay with phosphoglucumutase and glucose 6-phosphate dehydrogenase as described elsewhere [19]. Initial rates of the phosphorolysis and synthesis reaction were determined by using discontinuous assays under conditions reported previously [22] and by measuring the release of G1P and phosphate respectively. If not mentioned otherwise, starch was used as the α -glucan substrate. X1P (α -D-xylose 1-phosphate) was tested as a glucosyl

donor of the synthesis reaction catalysed by CcStP. Enzyme reactions were performed at 30 °C in 0.1 M sodium acetate buffer (pH 6.0), containing 10 mM potassium sulphate and 10 mM X1P. Maltopentaose was used as the xylosyl acceptor, and the concentration was adjusted to the corresponding K_m value of wild-type enzyme or mutant. Control reactions lacking maltopentaose were performed in all cases. Samples were taken after certain incubation times, and the concentration of phosphate in the sample was measured and corrected by the corresponding blank value. Apparent catalytic rates were calculated from the linear relationship of phosphate released against time. The inhibition by GL (D-gluconic acid 1,5-lactone) was analysed in the phosphorolysis direction using the assays described above. The release of G1P was measured at different concentrations of maltopentaose (1–50 mM), whereas the concentration of phosphate (50 mM) was saturating in the presence of different inhibitor concentrations.

Error hydrolysis

To determine if CcStP and the H334A mutant thereof show hydrolyase activity towards α -glucan substrates, we measured the release of glucose from maltopentaose in the absence of phosphate. The enzyme (0.2 mg/ml) was incubated at 30 °C in 50 mM triethanolamine buffer (pH 7.0) containing 70 mM maltopentaose and 15 mM potassium sulphate, which stabilizes the enzyme. Samples were taken after 1, 5 and 22 h, and the concentration of glucose was measured in a coupled assay with hexokinase (1 unit/ml) and glucose 6-phosphate dehydrogenase (2 units/ml) in 10 mM sodium phosphate buffer (pH 7.7), containing 4 mM magnesium sulphate, 1.6 mM ATP and 1.6 mM NAD⁺. The appropriate controls lacking maltopentaose or the enzyme were determined. With the chosen assay, it was possible to measure enzymic rates ≥ 0.0003 s⁻¹.

Equilibrium binding studies

Dissociation constants for enzyme complexes with GL and G1P were determined using data from fluorimetric titrations in which quenching of cofactor fluorescence served as reporter of the binding event. Fluorescence measurements were performed at 30 ± 1 and 10 ± 1 °C using an F4500 fluorescence spectrometer (Hitachi, Tokyo, Japan) and quartz cells of 1 cm optical path (Hellma, Muellheim, Germany). The photomultiplier voltage was set at 700 V. The excitation wavelength was 330 nm and the slit widths for excitation and emission were 5 nm each. Titrations were performed by adding successively increasing amounts of ligand to 600 μ l of the enzyme solution (5.5 μ M of 88 kDa protein subunits) in 50 mM triethanolamine buffer (pH 7.0). Fluorescence spectra of PLP were recorded in duplicate between 350 and 550 nm at every titration step. The dilution of the enzyme solution after adding ligand solutions did not exceed 20%. The relative change in fluorescence intensity of the enzyme-bound PLP, corrected by the appropriate dilution factor, was evaluated from the peak at 520 nm and the valley at 455 nm.

Stopped-flow kinetic measurements

Kinetic transients of GL binding to CcStP were measured by monitoring the quenching of PLP fluorescence using an SX-18MV-R stopped-flow spectrometer (Applied Photophysics, Leatherhead, Surrey, U.K.) equipped with a high-intensity xenon arc lamp and a 5 μ l cell compartment. The temperature of the syringes containing the reactants and the cell compartment was 10 °C. All measurements were performed in duplicate using the enzymes dissolved in 50 mM triethanolamine buffer (pH 7.0). The

excitation wavelength was 330 nm, and fluorescence emission was recorded with a cut-off emission glass filter (WG 475 nm). All fluorescence scans covered the wavelength range of 475–600 nm. The instrument settings were such that 80% of the photomultiplier voltage corresponded to cofactor fluorescence in the absence of ligands. The final enzyme concentration was constant at 5.5 μ M and the concentration of GL was varied as indicated. After rapid mixing of enzyme and the respective GL solution (which had been freshly prepared), the time course of cofactor fluorescence was determined, recording 1000 points per experiment.

Data processing

Data fitting was performed with the program Sigma Plot 2001 (SPSS, San Rafael, CA, U.S.A.) using unweighted least-squares regression methods. Initial rates (v), obtained under conditions in which the concentration of one substrate was varied and the level of the other substrate was constant, were fitted to eqn (2), where k_{cat} is the catalytic-centre activity, $[E]$ and $[S]$ are molar concentrations of phosphorylase monomer (88 kDa) and substrate respectively and K_m the apparent Michaelis constant for the substrate.

$$v = k_{cat}[E][S]/(K_m + [S]) \quad (2)$$

A preliminary analysis of inhibitor-binding data was performed by plotting reciprocal initial rates against reciprocal substrate concentrations. The data were fitted to the appropriate one of the following equations.

$$v = k_{cat}[E][S]/\{[S] + K_m(1 + [I]/K_{ic})\} \quad (3)$$

$$v = k_{cat}[E][S]/\{[S](1 + [I]/K_{iuc}) + K_m\} \quad (4)$$

$$v = k_{cat}[E][S]/\{[S](1 + [I]/K_{iuc}) + K_m(1 + [I]/K_{ic})\} \quad (5)$$

Equation (3), (4) or (5) was used when competitive, uncompetitive or mixed-type inhibition was observed respectively. K_{ic} and K_{iuc} relate to inhibitors that affect the slope or the intercept of the double-reciprocal plot respectively. $[I]$ is the inhibitor concentration.

Apparent dissociation constants (K_d) were obtained from non-linear fits of the data obtained from steady-state fluorescence titration to the equation

$$\Delta F = \Delta F_{max}[L]/(K_d + [L]) \quad (6)$$

where ΔF is the difference in fluorescence signal for the cofactor PLP in the absence and presence of ligand (L). ΔF_{max} is the difference at apparent saturation of the enzyme with ligand.

Stopped-flow fluorescence traces were fitted to the following equation:

$$\Delta F_t = \Delta F[1 - \exp(-k_{obs}t)] \quad (7)$$

where k_{obs} is the apparent first-order rate constant and ΔF_t the change in PLP fluorescence induced by ligand binding at time t , compared with the fluorescence at zero time when no ligand was present. As above, ΔF is the difference in fluorescence signal at equilibrium.

The dependence of k_{obs} on $[L]$ was analysed according to

$$k_{obs} = k_1[L] + k_{-1} \quad (8)$$

where k_1 and k_{-1} are the rate constants for association and dissociation of the ligand respectively.

Table 1 Apparent kinetic parameters in the direction of phosphorolysis and synthesis of the wild-type and mutant CcStP

Kinetic parameters were determined as described in the Experimental section; n.d., not determined.

	Wild-type	H334A	H334N	H334Q
Phosphorolysis				
$K_{\text{m starch}}$ (mM)	0.23 ± 0.01	0.25 ± 0.02	1.0 ± 0.1	0.32 ± 0.03
$K_{\text{m glycogen}}$ (mM)	1.6 ± 0.1	1.6 ± 0.1	16 ± 1	1.7 ± 0.1
$K_{\text{m maltopentaose}}$ (mM)	3.8 ± 0.5	3.5 ± 0.3	103 ± 6	4.1 ± 0.2
$K_{\text{m phosphate}}$ (mM)	6.8 ± 0.9	12 ± 1	13 ± 1	12 ± 1
$k_{\text{cat starch}}$ (s^{-1})	74 ± 1	80 ± 2	1.5 ± 0.1	0.52 ± 0.01
K_{ic} (mM)	0.041 ± 0.007	0.0081 ± 0.0027	n.d.	0.042 ± 0.003
K_{iuc} (mM)	0.099 ± 0.009	0.021 ± 0.004	n.d.	0.99 ± 0.17
Synthesis				
$K_{\text{m G1P}}$ (mM)	0.85 ± 0.05	1.8 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
$k_{\text{cat G1P}}$ (s^{-1})	52 ± 1	49 ± 2	1.27 ± 0.03	0.68 ± 0.02
$k_{\text{cat X1P}}$ (s^{-1})	$(2.6 \pm 0.1) \times 10^{-3}$	$(2.8 \pm 0.1) \times 10^{-3}$	$(4.8 \pm 0.4) \times 10^{-4}$	$(6.6 \pm 0.2) \times 10^{-3}$
R_{sel}^*	20000	17500	2600	100

* The selectivity ratio was calculated from $R_{\text{sel}} = k_{\text{cat G1P}}/k_{\text{cat X1P}}$.

RESULTS

Purification and structural characterization of wild-type and mutant CcStP

Recombinant wild-type and the mutants H334A, H334N and H334Q were expressed in *E. coli* and purified to apparent homogeneity in a yield of approx. 50 %. All enzymes contained approx. 0.8 PLP per each 88 kDa protein subunit. Mutants and the wild-type exhibited similar cofactor fluorescence emission spectra (excitation at 330 nm), which were recorded at 30 °C in 50 mM triethanolamine buffer (pH 7.0) containing 50 mM phosphate. CD spectra of wild-type and mutants were nearly superimposable (results not shown), indicating that the relative composition of secondary structural elements of the wild-type was not changed as a result of the mutations.

Kinetic analysis of wild-type and mutant CcStP

The specific activities in the direction of starch phosphorolysis of wild-type, H334A, H334N and H334Q were 30, 24, 0.42 and 0.26 units/mg respectively. pH effects on catalytic rates of wild-type and H334N mutant in the forward and reverse reaction were compared under conditions in which both substrates were saturating. The profiles of logarithmic rate versus pH were identical (results not shown). Initial rates of α -glucan phosphorolysis (p) and synthesis (s) by wild-type and the mutants were recorded using different substrates. Fits of the data to eqn (2) yielded steady-state kinetic parameters that are summarized in Table 1. Catalytic-centre activities $k_{\text{cat}}(\text{p})$ and $k_{\text{cat}}(\text{s})$ were strongly decreased in H334N and H334Q mutants, compared with the corresponding wild-type values. k_{cat} values of H334A and the wild-type were similar. According to $k_{\text{cat}}(\text{p})$ values, there was a similar preference of wild-type and the mutants to convert α -glucan substrates in the order: starch > glycogen > maltopentaose (results not shown). Comparison of K_{m} values for α -glucan substrates, which are effective dissociation constants [7] showed that replacement of His-334 by Asn, but not that by Ala or Gln, decreased the binding affinity (Table 1). Interestingly, the approx. 10-fold preference of the wild-type (and the H334A and H334Q mutants) for binding starch compared with binding maltopentaose was amplified in the H334N mutant by an additional factor of 10. K_{m} values for phosphate and G1P were not strongly affected in all mutants (~2-fold increase, compared with wild-type values).

We looked at the ability of wild-type and mutant enzymes to use X1P as glucosyl donor in place of G1P, and results are

Figure 2 Structures of the glucosyl oxocarbenium ion (A) and two structural analogues, GL (B) and NJT (C), with the proposed charge distribution in these compounds indicated

shown in Table 1. The wild-type displayed a very large selectivity ratio, $R_{\text{sel}} = k_{\text{cat G1P}}/k_{\text{cat X1P}} = 20000$. The value of R_{sel} was decreased by factors of approx. 8 and 200 in H334N and H334Q mutants respectively. Wild-type and H334A mutant did not show detectable hydrolase activity using maltopentaose as the substrate. Considering the limit of detection under the conditions used, this implies a rate of error hydrolysis that must be smaller than $\sim 2.5 \times 10^{-5}$ times ($= 74 \text{ s}^{-1}/0.0003 \text{ s}^{-1}$) the phosphorolysis rate.

GL inhibition of wild-type and mutant enzymes

Initial rates were recorded under conditions in which [phosphate] was saturating and [starch] was varied at ten suitable levels of GL over the range 2–1000 μM . The structure of GL is shown in Figure 2. Data were plotted as reciprocal rates versus reciprocal concentrations of starch and fitted to the appropriate equation (eqn 3, 4 or 5). Results are summarized in Table 1 and show that, in all the cases, the inhibition pattern was a mixed type (eqn 5) and the value of K_{ic} was smaller than the corresponding K_{iuc} value. H334A was more strongly inhibited by GL than the wild-type, reflecting approx. 5-fold decrease in both K_{ic} and K_{iuc} . The effect of the mutation H334Q was to increase K_{iuc} by approx. 10-fold compared with the wild-type value.

Table 2 Dissociation constants of CcStP–ligand complexes obtained by fluorimetric titrations

K_d (μ M) values were determined from non-linear fits to eqn (6) of fluorescence titration data recorded at $30 \pm 1^\circ\text{C}$. Values determined at $10 \pm 1^\circ\text{C}$ are given in parentheses.

	Wild-type	H334A	H334N	H334Q
$K_{d\text{GL}}$	260 ± 20 (100 ± 10)	7 ± 0.5	5.2 ± 0.5	6.1 ± 0.4 (12 ± 0.7)
$K_{d\text{GL}}^*$	15 ± 1 (10 ± 0.5)	17 ± 0.1	510 ± 20	840 ± 50 (500 ± 20)
$K_{d\text{G1P}}$	700 ± 50	15 ± 0.5	85 ± 8	64 ± 7

* Data were obtained in the presence of 50 mM phosphate. Results are means $\pm$ S.E.M. for three independent experiments.

Figure 3 Correlation between catalytic rates and the reciprocal dissociation constants for GL binding to enzyme–phosphate complexes of wild-type CcStP and His-334 mutants

K_d values were determined by steady-state fluorescence titration (see Table 2). k_{cat} values of starch phosphorylase are taken from Table 1. The solid line represents a linear fit to the data.

Steady-state analysis of ligand binding

We studied binding of GL and G1P to wild-type and mutant enzymes using fluorescence titration experiments at 10 and 30°C . Data were fitted to eqn (6), and dissociation constants $K_{d\text{GL}}$ and $K_{d\text{G1P}}$ are summarized in Table 2. Values of $K_{d\text{G1P}}$ were decreased 8–50-fold in the mutants, compared with wild-type, indicating that binding of G1P to free enzyme was tightened significantly by replacements of the side chain of His-334. Comparison of $K_{d\text{GL}}$ values for the wild-type determined under conditions in which phosphate was absent or present at a saturating level revealed that the binding affinity for GL was enhanced 17-fold for the enzyme bound to phosphate. Replacement of His-334 resulted in an approx. 40-fold strengthened binding of GL to the free enzyme, the decrease in $K_{d\text{GL}}$ compared with the corresponding wild-type value being independent of the side chain introduced by the mutation. An interesting observation made with the mutant was that, unlike the wild-type, there was a large increase in $K_{d\text{GL}}$ when [phosphate] was saturating, compared with the $K_{d\text{GL}}$ value determined in the absence of phosphate. The change in $K_{d\text{GL}}$ followed the order: H334A (3-fold) < H334N (100-fold) < H334Q (140-fold).

Figure 3 shows a log-log correlation of the catalytic-centre activities for phosphorylase of starch with the reciprocal dissociation constants for GL binding to enzyme–phosphate complexes of the wild-type and the mutants (Table 1). The data were fitted to a straight line, yielding a slope value of 1.19 ± 0.07 and an excellent coefficient of determination ($r^2 = 0.991$). Figure 3 establishes an LFER (linear free-energy relationship), which indicates that structural alterations in the active sites of the mutants produce parallel effects of a similar magnitude on the

catalytic rate and GL binding. By way of comparison, a log–log correlation of k_{cat}/K_m with $1/K_{d\text{GL}}$ was also linear (slope = 1.35 ± 0.18), however, with a smaller value of r^2 ($= 0.946$). A plot of $\log K_m$ versus $\log 1/K_{d\text{GL}}$ did not show a useful correlation of the two parameters. Figure 3 includes data for a mutant of CcStP (R242A) that harbours the side-chain replacement at a site near or at the dimer interface and remote from the active site. The structural properties of R242A have recently been reported [18]. The finding that mutations at the active site and mutations distant from it can be placed into a single log–log correlation of k_{cat} versus $1/K_{d\text{GL}}$ suggest that the LFER in Figure 3 is general.

Presteady-state analysis of GL binding

Kinetic transients of GL binding to wild-type and H334Q mutant were recorded in rapid-mixing stopped-flow experiments at 10°C measuring the quenching of PLP fluorescence. The stopped-flow traces monitored at different levels of GL and fits of the experimental time courses to eqn (7) are shown in Figures 4(A)–4(C). In the absence of phosphate, the change in PLP fluorescence induced by GL binding to H334Q was significant but too small to permit kinetic analysis of the stopped-flow traces (results not shown). Plots of the pseudo-first-order rate constants for the kinetic transient against the GL concentration were linear, with a non-zero intercept (Figure 5). The results imply a single-step mechanism of GL binding: $E + \text{GL} \leftrightarrow E - \text{GL}$. Fits of the data to eqn (8) yielded slope and intercept values (Table 3), which correspond to the rate constants for GL association (k_1) and dissociation (k_{-1}) respectively. The relationship $K_{d\text{GL}} = k_{-1}/k_1$ was used to calculate thermodynamic parameters, which agree well with $K_{d\text{GL}}$ values determined from equilibrium binding data recorded in steady-state fluorescence titrations (Table 2) and stopped-flow experiments (Figure 5, Table 3). The results show that, in the wild-type, tighter binding of GL to enzyme–phosphate when compared with free enzyme results from a 2.4-fold increase in k_1 and a 2.5-fold decrease in k_{-1} . Under conditions of saturation in phosphate, tighter binding of GL to H334Q when compared with the wild-type is entirely due to a 50-fold decrease in k_{-1} .

DISCUSSION

Figure 1, based on X-ray crystallographic evidence [10,11], summarizes the proposed roles of His-334 in the mechanism of α -retaining glucosyl transfer catalysed by α -glucan phosphorylases. In the present study, we have used a mutational approach to probe the function of the side chain of His-334 and provide a comparative reaction co-ordinate analysis for three site-specific mutants and the wild-type. The maintenance of native-like secondary structure and substrate binding properties of wild-type CcStP in all mutants suggests that interpretations of the kinetic consequences of each mutation can be based on the assumption that the enzyme active site has not been globally disrupted by site-directed replacement of the original side chain with the side chain of alanine, asparagine or glutamine.

Mutations of His-334 affect ground-state interactions of the sugar in subsite – 1

Results of reversible inhibitor-binding studies using kinetic measurements and fluorescence titrations allow us to identify two distinct effects of the site-specific replacements of His-334 on ground-state interactions of the sugar in subsite – 1: changes in Gibbs free energy of binding ($\Delta\Delta G_b$) at the binary complex level; and at the level of the ternary complex where direct or enzyme-mediated interactions between the ligands are altered as a result of the mutations.

Figure 4 Presteady-state progress curves of GL binding to wild-type CcStP in the absence (A) and presence (B) of 50 mM phosphate and to the H334Q mutant (C) in the presence of 50 mM phosphate

Stopped-flow experiments were performed at 10 °C in 50 mM triethanolamine buffer (pH 7.0). The enzyme (5.5 μM) was mixed with 20–1250 μM (A), 2.5–100 μM (B) and 50–5000 μM (C) GL, as indicated. The quenching of cofactor fluorescence was recorded as described in the Experimental section. The solid lines represent fits of the data to eqn (7).

Using the relationship $\Delta\Delta G_b = -RT \ln(K_{dGIP}^{wt}/K_{dGIP}^{mut})$, where R is the gas constant, T the temperature in K, wt stands for wild-type and mut for mutant, we use the data in Table 2 to calculate that complexes of G1P and unligated mutant enzymes are stabilized by up to 9.7 kJ/mol, relative to the same complex of the wild-type. K_{mGIP} values in Table 1 are G1P dissociation constants under conditions in which the α -glucan is bound to subsites +1 to +4 and perhaps another polysaccharide binding site in CcStP [22]. In the wild-type, the K_{mGIP} is identical with K_{dGIP} , indicating that the binding affinity of G1P is not dependent on the presence or absence of α -glucan. The same comparison performed for the mutants shows that binding of G1P to the complex of enzyme and α -glucan is weaker than that to the free enzyme. The interaction coefficient α which reflects how binding of α -glucan influences the affinity of the enzyme for G1P is given by the

Figure 5 Dependence on GL concentration of the first-order rate constant (k_{obs}) for the kinetic transient of GL binding to the wild-type (●) and the H334Q mutant (○) in the absence (A) and presence (B) of 50 mM phosphate

The solid line represents a fit of the data to eqn (8).

Table 3 Rate constants of GL binding to wild-type and mutant CcStP

k_1 and k_{-1} were obtained from linear fits of k_{obs} values to eqn (8) (Figure 5).

	k_1 (mM ⁻¹ · s ⁻¹)	k_{-1} (s ⁻¹)	K_d (μM)*	K_d (μM)†
Wild-type	8.3 ± 0.1	0.70 ± 0.02	85 ± 6	90 ± 8
Wild-type‡	19.5 ± 0.3	0.28 ± 0.03	14 ± 0.5	14 ± 1
H334Q‡	22.5 ± 1.3	14.7 ± 0.7	650 ± 40	340 ± 17

* $K_d = k_{-1}/k_1$.

† Experimental K_d was determined by fitting ΔF values to eqn (6).

‡ Data were obtained in the presence of 50 mM phosphate. Rate constants are means ± S.E.M. for two independent experiments.

ratio of K_{mGIP} and K_{dGIP} . Thus the calculated α values increase in the order, wild-type (~1), H334Q (15), H334N (15) and H334A (100). Therefore, in contrast with the wild-type, where binding of G1P and α -glucan occurs independent of one another, the mutations induce a selective destabilization of the central ternary complex primed for α -glucan synthesis, relative to the binary complex of enzyme and G1P. The side chain of His-334 thus influences the internal equilibrium of enzyme-bound forms at the steady state in the reverse direction of the reaction (eqn 1).

The side chain of His-334 is required for transition state stabilization

Kinetic consequences of replacing His-334 by asparagine and glutamine are reflected by decreases in a similar magnitude in the values of k_{cat} and k_{cat}/K_m . Therefore the mutations appear to destabilize primarily the transition state of enzymic glucosyl transfer and have smaller effects on ground-state binding of the reactants. The value of k_{cat}/K_m presents an extrapolation to conditions in which the substrate is limiting and therefore does not contain ground-state effects that are seen together with transition-state effects in the value of k_{cat} which is an extrapolation to infinite substrate levels. The site-specific substitution in H334N or H334Q

is one that exchanges a side chain that potentially ionizes over the pH range 5–8 by another that does not. Interestingly, therefore, the pH profiles of $\log k_{\text{cat}}$ in the forward and reverse directions of the reaction were identical for the wild-type and H334N mutant.

The H334A mutant retained most of the catalytic proficiency of the wild-type, expressed in either k_{cat} or k_{cat}/K_m terms. This result suggests that another enzyme group or water partly occupies the position vacated by the substitution of the original side chain of His-334 with the side chain of alanine and effectively replaces it in catalysis. Considering a scenario of water bound nearby the reactive carbon in subsite – 1, it is noteworthy that the H334A mutant catalyses glucosyl transfer without a detectable error in hydrolysis [16]. Interestingly, retaining the GT-A fold GTs of families GT-6 and GT-8 display significant hydrolase activity towards their nucleotide-activated glycosyl donor substrates. The ratio of catalytic efficiencies for the transferase and hydrolase reaction can be changed significantly through site-directed replacements of putative active-site residues [23,24].

Mutations of His-334 cause a large change in donor substrate selectivity for the reverse direction of the reaction (eqn 1). The improved utilization of X1P, relative to G1P, in H334N and H334Q mutants may be partly explained by the loss of hydrogen-bonding interactions with the C-6 hydroxy group of G1P in the mutants (see Figure 1). The extremely low activity of all enzymes for reaction with X1P may be due to the additional loss of a hydrogen bond mediated by Asn-449 of *EcMalP* [10] (Asn-437 in *CcStP*) on switching from glucosyl to xylosyl donor substrate; or it may reflect the inability of the enzymes to stabilize equally well the different conformations of glucosyl (half-chair) and xylosyl (half-chair or boat) oxocarbenium ions in the transition state of the reaction (see [25,26] for a general discussion of conformational itineraries of GHs and GTs). Finally, an electrostatic stabilization of the transition state derived from the appropriately disposed C-6 hydroxy group seems to be possible. The relationship, $\Delta\Delta G^\ddagger = -RT \ln 20000 (=k_{\text{cat,G1P}}/k_{\text{cat,X1P}})$, can be used to calculate that approx. 25 kJ/mol less binding energy ($\Delta\Delta G^\ddagger$) is available to stabilize the transition state of the reaction with X1P, compared with that of the reaction with G1P. Using rabbit muscle GP, Street et al. [27] measured an identical $\Delta\Delta G^\ddagger$ value on comparing reactions with G1P and the 6-deoxy analogue thereof.

Structure–function relationships with other GTs

The robust enzyme activity seen in His-334 mutants of *CcStP* is not completely unexpected considering that retaining GTs may have evolved in different ways to promote and stabilize the oxocarbenium ion-like transition states of their reactions. *E. coli* trehalose 6-phosphate synthase OtsA of family GT-20 (GT-B fold) has a histidine residue in an identical position as His-334 [28]. Studies by molecular modelling and site-directed mutagenesis [29] have indicated that *E. coli* glycogen synthase (family GT-5, GT-B fold) may share considerable active-site similarity with other GT-B fold GTs. In particular, His-161 of the glycogen synthase is predicted to have the same function as His-334 of *CcStP*. Outside the GT-B fold GTs, the lipopolysaccharyl α -1,4-galactosyltransferase LgtC from *Neisseria meningitidis*, a member of the family GT-8 (GT-A fold), possesses a glutamine residue (Gln-189) in place of the histidine and positions the side-chain-derived carbonyl group above the sugar ring, close to the C-1–O-5 bond [30]. Replacement of Gln-189 by an aspartate residue decreased k_{cat} to 3 % of that of the wild-type enzyme, and a significant fraction of the total mutant enzyme contained covalently bound galactosyl moiety [24]. Interestingly, the site of labelling was not Asp-189 but the nearby Asp-190. X-ray structures of glycogenin (family GT-8) [31] and α -1,3-galactosyltransferase (family GT-6) [32],

which, like LgtC and glycogenin, displays a GT-A fold, have revealed additional residues that may have a role in catalysis by these GTs. In the family GT-64 (GT-A fold), the guanidinium group of Arg-293 of α -1,4-*N*-acetylhexosaminyltransferase is supposed to provide stabilizing interactions with the reactive C-1 from above the sugar ring [33]. The current evidence does not provide clear answers to outstanding questions regarding the catalytic mechanism of retaining GTs. It emphasizes, however, that the search for catalytic residues in GTs must consider the possibility of large substrate-induced protein conformational changes that may be required during catalysis to bring the active-site groups into the appropriate positions (see e.g. [10,11]).

The complex of enzyme, phosphate and GL bears partial transition state analogy

The common use of GL as reversible inhibitor of GTs reflects the idea that the reactions catalysed by members of this enzyme class involve transition states with substantial oxocarbenium ion character. Compounds that mimic the sp^2 -hybridized anomeric centre and, consequently, the partially planar conformation of the glucopyranosyl ring, and the build-up and distribution of the positive charge of the oxocarbenium ion in the transition state should therefore be good inhibitors of the enzymes. The partial analogy of GL with the glucosyl oxocarbenium ion is shown in Figure 2.

In the wild-type, GL binding to the complex of enzyme and phosphate is approx. 10-fold tighter than to the free enzyme. This synergism has previously been observed in kinetic and structural studies of rabbit muscle GP [34,35] and *EcMalP* [14]. Our results are novel since they provide a kinetic mechanism for GL binding and the synergistic interaction at the ternary complex level. Binding of GL occurs in a single-step process, and the increased affinity of the enzyme for GL when phosphate is bound reflects changes in microscopic rate constants for GL on-rate (increase) and off-rate (decrease) by approximately the same factor.

Mutations of His-334 by alanine, asparagine and glutamine residues revert ligand synergism seen in the wild-type such that binding of phosphate now weakens the binding of GL in all three mutants. The 50-fold faster GL off-rate in the H334Q mutant explains differences in the equilibrium dissociation constants for ternary complexes of the wild-type and this mutant. Interestingly, however, binding of GL to unligated enzyme was stronger in the mutants than in the wild-type, indicating that, on replacement of His-334, binding of one ligand to form a binary complex in the ground state fails to induce a correct recognition site for the other ligand. Considering crystallographic evidence for rabbit muscle GP discussed later [36], we favour a scenario in which a conformational change triggered by GL binding in the wild-type, but not to the same extent in the mutants, is required for the proper alignment of residues that bind phosphate productively. This structural rearrangement is expected to utilize binding energy derived from the protein–ligand interactions, causing the observable K_d to increase. Therefore it could well be responsible for the different stabilities of binary complexes of wild-type and mutants with GL.

Results obtained with wild-type and mutant enzymes reveal a linear correlation between $\log k_{\text{cat}}$ (and $\log k_{\text{cat}}/K_m$) for the enzymic phosphorylation of starch and $\log 1/K_d$ for GL binding to enzyme–phosphate with a good coefficient of determination. In contrast, no useful correlation exists across the same series of enzymes between steady-state kinetic parameters and $\log 1/K_d$ for GL binding to the free enzyme. The LFER analysis rigorously supports the notion that GL in combination with phosphate but not in the absence thereof exhibits properties consistent with a partial transition state analogue. It does not, however, measure how well the inhibitor complex approximates the transition state (see

[37] for a general discussion). A plot of $\log K_m$ versus $\log 1/K_d$ for GL binding to enzyme–phosphate does not show any correlation between the two parameters, stressing the absence of ground-state mimicry provided by the combined binding of GL and phosphate. The similar slope values seen in the correlations of $\log k_{cat}$ and $\log k_{cat}/K_m$ reinforce this conclusion. An important requirement for the analysis to be valid is that the chemical step of the enzymic reaction is rate-limiting for the wild-type enzyme. It seems to be fulfilled for glucosyl transfer to phosphate catalysed by phosphorylase [7,38].

The ligand binding constants determined through steady-state kinetic analysis of the inhibition of enzymic rates agree with previous reports showing that GL is a mixed-type inhibitor of CcStP and the mutants thereof [15,34,35]. The results corroborate the notion that GL binding to phosphorylase–phosphate occurs in two ways: one that excludes binding of the α -glucan and another in which both GL and α -glucan are bound [15,34,35,38]. Binding of GL in subsite –1 (which probably produces the quenching of PLP fluorescence) should be compatible with α -glucan binding in subsites +1 to +4. Direct comparison of K_{iuc} with K_d derived from fluorescence titration data is, however, complicated because the prevailing ligand complexes are not the same under the experimental conditions used.

Interpretations of X-ray structures confirmed by biochemical data

Our results are in good agreement with previous X-ray crystallographic studies of other GPs. Mitchell et al. [36] report synergistic binding of the stable glucosyl oxocarbenium ion mimic NJT (nojirimycin tetrazole; Figure 2) and phosphate to rabbit muscle GP. They provide a structural basis of how one ligand promotes the binding of the other and identify interactions of His-377 (His-334 in CcStP) in NJT with the binary enzyme–inhibitor complex and the ternary complex with phosphate. A series of small conformational changes are observed when phosphate binds to enzyme–NJT whereby the side chain of Arg-569 (Arg-523 in CcStP) undergoes the greatest shift to a position that enables interactions with two oxygens of the phosphate ligand. Coupled with the rearrangement of the guanidinium group of Arg-569, the side chain of Asn-284 (Thr-249 in CcStP) that interacts with O-2 of the inhibitor in the binary complex moves away to allow polar contacts between a phosphate oxygen and the O-2 hydroxy group of the glucopyranose ring. In addition, electrostatic contacts are made between a phosphate oxygen and N-1 of NJT (which is in a position formally analogous to O-5 of a glucosyl oxocarbenium ion). The contacts made by the oxygen of the main chain carbonyl group and the side chain of His-377 are essentially those depicted in Figure 1 and independent of the absence and presence of phosphate. van der Waals contacts of His-377 with C-1 and N-1 of NJT are also similar in both complexes. At the level of the ternary complex, however, they involve contacts between the same positions on the inhibitor and phosphate. The absence of exactly these interactions in which His-377 and phosphate are disposed to ‘sandwich’ the C-1 and O-5 atoms of the glucosyl oxocarbenium ion (or the analogous atoms of an inhibitor mimicking it) might partly explain why synergistic binding of GL and phosphate is sensitive to side-chain replacements of the histidine residue.

Conclusions

Mutational studies of CcStP confirm the notion [10] that His-334 assists (but is not essential) in the catalysis of GP. Well-balanced contacts made by the side-chain and main-chain carbonyl group of the histidine are required for optimum transition state stabilization, and this is shown to involve the precise positioning of

a glucosyl oxocarbenium ion and the anionic phosphate leaving group/nucleophile, relative to each other. In addition, His-334 seems to influence the internal equilibrium of reactant states in the kinetic mechanism of the enzyme. The present results are consistent with the most widely accepted mechanism for GPs in which leaving group departure and nucleophilic attack occur in a concerted but asynchronous manner on the same (α) side of the glucoside [9,10,36]. They do not provide novel answers to the debate as to whether the catalytic mechanism of GPs involves some form of a covalent intermediate. Other authors [9,36] have discussed the possibility that the backbone amide carbonyl of His-334 could function as a nucleophile in a manner similar to that observed in the neighbouring group participation of the C-2-acetamide group in *N*-acetyl glycosaminide substrates displayed by some hexosaminidases (family GH-20) [39], hyaluronidases (family GH-56) [40] and chitinases (family GH-18) [41,42]. However, currently, there is no convincing support for this mechanism in GPs.

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