pH-dependence of the fast step of maltose hydrolysis catalysed by glucoamylase G1 from *Aspergillus niger*

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The presteady-state kinetic parameters of the interaction of wild-type glucoamylase from *Aspergillus niger* (EC 3.2.1.3) with maltose were obtained and analysed in the pH range 3–7 with intervals of 0.25 pH units. In all cases the following three-step reaction scheme was found to apply.

The general result of the analysis of the presteady-state kinetics is that glucoamylase G1 is affected by the protonation states of three groups, with pK_a values of 2.7, 4.5 and 5.7 in the free enzyme and of 2.7, 4.75 and 6.5 in the first enzyme–substrate complex. The protonation of the group in the enzyme–substrate complex with a pK_a 6.5 had no effect on k_2 (1640 s^{−1}) or k_{-2} (20 ± 4 s^{−1}), but resulted in a stronger enzyme–substrate interaction, due to a decrease of K_1 from 40 to 6.3 mM. In other

words, when the substrate is bound, the pK_a of the acid group changes to increase the fraction of reactive enzyme. Since this pK_a parallels that of the Michaelis complex, known from the pH-dependence of k_{cat} , the group in question is most probably the catalytic acid Glu-179. Protonation of Glu-179 thus is of no importance in the second step, clearly indicating that this step represents a conformational change and not the actual hydrolysis step of the reaction. Protonation of the pK_a = 4.75 group leads to a small decrease in k_2 to 1090 s^{−1}, and also to minor changes in K_1 . The group with pK_a = 2.7 leads to a major decrease of k_2 , of which the limit may be zero, but shows no effect on K_1 . Thus no difference is seen between the pK_a values of the free enzyme and of the first enzyme–substrate complex at low pH.

Key words: glucoamylase reaction mechanism, presteady-state kinetics.

INTRODUCTION

Glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) is an inverting exoglycosidase, producing β -D-glucose by hydrolysis of α -1,4-glucosidic bonds, and of α -1,6-bonds with much less efficiency, from the non-reducing end of starch and related oligo- and polysaccharides [1,2]. Glucoamylases from *Aspergilli* exist in two natural forms: G1 and G2. The G1 form from *Aspergillus niger* (Ala-1–Arg-616) consists of a catalytic and a starch-binding domain connected with a highly O-glycosylated linker region.

The three-dimensional structure of the catalytic domain and part of the linker region of glucoamylase from *A. awamori* var. X100 is known [3–8]. The sequences of the glucoamylases from *A. awamori* var. X100 and *A. niger* show 95% amino acid identity and the catalytic sites of the two glucoamylases show no differences [8a].

The proposed and generally accepted mechanism of hydrolysis involves proton transfer to the glycosidic oxygen of the scissile bond from a general acid catalyst, formation of an oxocarbenium ion-like transition state and a nucleophilic attack of water assisted by a general base catalyst [9–12]. Glu-179 and Glu-400 have been identified as, respectively, the general acid and the general base catalysts and the pH-dependencies of steady-state kinetic parameters are in accordance with a rate-determining hydrolysis involving these two catalytic residues [7,13–16].

We have undertaken presteady-state kinetic studies of the mechanism of the *A. niger* enzyme and mutants thereof [8a,17–19] and have found that analysis according to a three-step reaction mechanism (model 1, shown here) of substrate catalysis involving two intermediates showed excellent compatibility:

It has been shown that the presteady-state kinetic results [19] are not in accordance with the classical model [2,21,22] of glucoamylase-catalysed reactions, which involves strong non-productive binding of substrates and intrinsic catalytic-constant values independent of the substrate length.

Figure 1 shows a stereoview of the active site of glucoamylase with bound D-glucodihydroacarbose. Mutations on the β -flank (as defined in Figure 1) primarily affect the second reaction step [8a]. We interpreted the step as a conformational change, where the substrate reaches the correct position for catalysis after the initial association, in which apparently the formation of hydrogen bonds to Arg-305 and to Glu-180 play important roles [8a]. This is also in accordance with a critical role of Glu-180 for the induction of a productive conformation of isomaltose [23].

We have seen further that mutations on the α -flank result primarily in almost total loss of catalytic turnover, $k_{cat} \rightarrow 0$, and have interpreted this as the lack of correct position of Glu-179 for catalysis in the second reaction step in these mutants. In contrast, it has been suggested that the fast second reaction step is the actual hydrolysis step and that the rate-determining step is a product-release step [24–26]. Clearly these conflicting conclusions need to be resolved. We have therefore studied the pH-dependencies of the presteady-state kinetic parameters in order to determine first of all whether or not the second reaction step shows a pH-dependence corresponding to that expected for a hydrolysis step.

EXPERIMENTAL

Enzymes and substrates

Glucoamylase G1 purified from the commercial preparation (AMG 200L) from Novo Nordisk A/S (Bagsvaerd, Denmark)

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Figure 1 Stereoview of the active site of glucoamylase from *A. awamori* var X100 with bound D-glucose-dihydroacarbose [8]

The four rings of the substrate are marked a, b, c and d. Hydrogen-bond interactions less than 3.0 Å are represented by dashed lines, that of Trp-52–Glu-179 (3.04 Å [8]) therefore does not appear. We define the α -flank of the active site as that on the right side of the ligand and the β -flank as that on the left side in this representation. Wat, water.

using affinity chromatography on acarbose–Sephacrose, as described in [27], and separated from the G2 form by FPLC chromatography on High-Load Q–Sephacrose, as described in [28], was a gift from Birte Svensson, Carlsberg Laboratory, Copenhagen, Denmark. Protein concentrations were determined by amino acid analysis [29]. Maltose was from Merck (Darmstadt, Germany). D-Glucose oxidase kits for glucose determination were obtained from Sigma (St. Louis, MO, U.S.A.).

Steady-state kinetics

Initial rates of hydrolysis of the maltose were determined at 8 °C in 0.05 M phosphate buffers, pH 6–7, using seven substrate concentrations in the range $0.125 \times K_m$ – $8 \times K_m$. The final enzyme concentrations were 0.1–1 μ M. Glucose released was determined by the glucose oxidase method using microtitre plates [16,30,31].

Stopped-flow fluorescence kinetics

Changes of intrinsic protein fluorescence as a result of ligand binding to glucoamylase in the presteady-state phase of the reactions were observed as described previously [8a,17–19]. Series of experiments were performed using 10 concentrations of maltose in an appropriate range dependent on the pH at 8 °C in 0.1 M sodium acetate buffers, pH 3–6.3 and 0.05 M phosphate buffers, pH 6–7, with 0.25-pH-unit intervals in a Hi-Tech Scientific PQ/SF-53 spectrofluorimeter equipped with a high-intensity xenon arc lamp. The excitation wavelength was 280 nm, the slit width 5 nm. The light emitted from the reaction mixture passed a cut-off emission filter (WG 320; 80 % transmittance at 320 nm). The final enzyme concentrations were in the range of 1.0–1.2 μ M. After rapid mixing of enzyme and substrate (less than 1 ms), the time course of the intrinsic fluorescence intensity (arbitrary units, V) was recorded. In total, 400 pairs of data were taken and data from 2–4 experiments were averaged. The averaged stopped-flow data were fitted to several non-linear analytic equations using the Hi-Tech HS-1 Data Pro software. In all cases the single-exponential expression (eqn. 1) resulted in the best fit to the stopped-flow trace. The traces were similar to those

obtained previously with the wild-type and mutants of *A. niger* glucoamylase [8a,17–19].

$$\Delta F([S], t) = \Delta F([S], \infty) [1 - \exp(-k_{\text{obs}} \times t)] \quad (1)$$

where k_{obs} is the observed first-order rate constant and $\Delta F([S], t)$ is the relative fluorescence change observed at the actual substrate concentration, [S], and time, t . $\Delta F([S], \infty)$ is the equilibrium change in fluorescence at a time, t , where no (or only negligible) hydrolysis has yet occurred. In no case have changes of the fluorescence corresponding to the pre-equilibrium of the first step in the reaction been observed. The usual control experiments, where the sum of the initial measured fluorescence of an enzyme–buffer mixture and a substrate–buffer mixture is compared with that obtained at zero time from the fit to eqn. (1) of the enzyme–substrate mixture in question, always showed identical values. According to model 1, when no initial change of fluorescence associated with ES_1 is observed, the fluorescence change is proportional to the concentration of ES_2 at any given time. Its final steady-state value at concentration [S] of the substrate, $\Delta F([S], \infty)$, is a hyperbolic function of [S] with the limit value $\Delta F_{\text{max}}([S], \infty)$, which is the maximal relative fluorescence change at saturation with the substrate, and with characteristic half-saturation substrate concentration, K_m [19]. The concentration-dependence of k_{obs} in all cases corresponded with the reaction scheme model 1 and provided values of K_1 , k_2 and k_{-2} from eqn. (2):

$$k_{\text{obs}} = \{k_2 / (1 + K_1/[S])\} + k_{-2} \quad (2)$$

The k_{-2} values obtained were almost constant, $20 \pm 4 \text{ s}^{-1}$.

Data analysis

The presteady-state and steady-state kinetic parameters were obtained from fits of the values of k_{obs} , $\Delta F([S], 4)$ and initial rates as functions of the substrate concentration as described previously [8a]. The pH-dependencies of the parameter values obtained were similarly analysed using the non-linear least-squares fitting software GraFit from Erithacus Software or ENZFITTER from Elsevier BIOSOFT to fit the appropriate equations given below. Double-logarithmic plots were not used.

RESULTS

Two carboxylic groups, those of Glu-179 and Glu-400, have been assigned as general acid and general base catalysts, respectively, in the assumed rate-determining hydrolytic reaction step of glucoamylase-catalysed reactions. These results based on pH-dependencies of steady-state kinetic parameters with pK_a values of ≈ 6 for Glu-179 and ≈ 3 for Glu-400 [14–16], chemical-modification studies [13] and structural data [7]. Here the pH-dependencies of the presteady-state kinetic parameters of the reaction of glucoamylase G1 with maltose were determined.

Figure 2 illustrates the presteady-state results obtained at pH 7, with $k_{\text{obs}} - k_{-2}$ (eqn. 2) shown as a function of the maltose concentration. Similar saturation curves were obtained at all the pH values investigated. The resulting values of the parameters at pH 7 ($0.1 \mu\text{M}$ $[\text{H}^+]$) obtained from the fit to eqn. (2) were: $K_1(\text{H}^+_{0.1 \mu\text{M}})$ 33 mM and $k_2(\text{H}^+_{0.1 \mu\text{M}})$ 1640 s^{-1} . The $K_1(\text{H}^+)$ value decreases in the pH range 7–4 and then becomes constant. At pH 4–3 $K_1(\text{H}^+_{0.1-1 \text{ mM}})$ was 3.8 mM, whereas the $k_2(\text{H}^+)$ value was constant in the range pH 7–6, $k_2(\text{H}^+_{0.1-1 \mu\text{M}}) = 1640 \text{ s}^{-1}$, and then decreased.

Scheme 1 illustrates the presteady-state reaction (the formation of ES_2 of model 1); just one protonation of the enzyme is taken into consideration. For Scheme 1 the experimentally obtained

Figure 2 Presteady-state kinetic results of the reaction of glucoamylase G1 with maltose at pH 7

$k_{\text{obs}} - k_{-2}$ is shown as a function of maltose concentration. The curve is that obtained from fitting eqn. (2) to the data with the resulting values of the parameters $K_1 = 33 \text{ mM}$ and $k_2 = 1640 \text{ s}^{-1}$.

Scheme 1 Reaction scheme illustrating the presteady-state part of the glucoamylase mechanism taking into consideration one protonization of an enzymic group

values of $k_2(\text{H}^+)$ and $K_1(\text{H}^+)$ are functions of the H^+ concentration, as described in eqns (3) and (4) [32]:

$$k_2(\text{H}^+) = k_{2,\text{E}} + (k_{2,\text{EH}} - k_{2,\text{E}})/(1 + K_{\text{ESH}}/\text{H}^+) \quad (3)$$

which states that $k_2(\text{H}^+)$ changes from the $k_{2,\text{E}}$ level to the $k_{2,\text{EH}}$ level and follows a saturation curve as H^+ increases from $\text{H}^+ \ll K_{\text{ESH}}$ to $\text{H}^+ \gg K_{\text{ESH}}$. If $k_{2,\text{E}}$ is known, the difference $[k_2(\text{H}^+) - k_{2,\text{E}}]$ shows a simple saturation curve as a function of the H^+ concentration (eqn. 3) from which K_{ESH} may be determined. Similarly,

$$K_1(\text{H}^+) = K_{1,\text{E}} + (K_{1,\text{EH}} - K_{1,\text{E}})/(1 + K_{\text{ESH}}/\text{H}^+) \quad (4)$$

Since, further, a relation exists between the K values (eqn. 5), all the parameters that describe the reaction of Scheme 2 may be obtained from an analysis using eqns (3–5).

$$K_{1,\text{E}} \cdot K_{\text{ESH}} = K_{1,\text{EH}} \cdot K_{\text{EH}} \quad (5)$$

The $k_2(\text{H}^+)$ values obtained here decrease with increasing H^+ concentration, the $k_{2,\text{E}}$ value thus is larger than the $k_{2,\text{EH}}$ value, and the positive difference $[k_{2,\text{E}} - k_2(\text{H}^+)]$, the saturating value of which is the positive difference $(k_{2,\text{E}} - k_{2,\text{EH}})$, was analysed. Since $k_2(\text{H}^+)$ is constant in the range pH 7–6, the value of $k_{2,\text{E}}$ is known to be 1640 s^{-1} . The result of the fit is shown in Figure 3. The fitted curve yielded: $k_{2,\text{EH}} = 1090 \text{ s}^{-1}$, $K_{\text{ESH}} = 18.8 \mu\text{M}$ and $pK_a = 4.73$. Values obtained at a pH lower than 4.25 did not fit this curve, indicating that a second protonation occurs at pH less than 4. Fitting of eqn. (3) using the value 1090 s^{-1} as $k_{2,\text{E}}$ of this second protonation resulted in a second K_{ESH} of 2.2 mM, with $pK_a = 2.7$ and a final $k_{2,\text{EH}}$ value ≈ 0 , as illustrated in Figure 4. These last values are rather poorly determined with standard errors $\approx 30\%$, and would have been obtained with greater confidence had the enzyme been stable at a pH lower than 2.75, where it tended to aggregate after 10–15 min.

$K_1(\text{H}^+)$ immediately decreases with increasing $[\text{H}^+]$ from pH 7, thus a $K_{1,\text{E}}$ value is not known, and the three parameters, $K_{1,\text{E}}$, $K_{1,\text{EH}}$ and K_{ESH} , were determined directly from fitting eqn. (4) to the data (Figure 5) in the pH range 7–5.5. The fitted curve yielded: $K_{1,\text{E}} = 40 \text{ mM}$, $K_{1,\text{EH}} = 6.3 \text{ mM}$ and $K_{\text{ESH}} = 0.3 \mu\text{M}$, with $pK_a = 6.5$, and thus (eqn. 5) $K_{\text{EH}} = 1.9 \mu\text{M}$ ($pK_a = 5.7$). Data in the pH range 2.75–5.25 did not fit this curve, indicating an influence of the group with pK_a 4.73 known from the analysis of the $k_2(\text{H}^+)$ results. Using the known final $K_{1,\text{EH}}$ value (3.8 mM) of this second protonation reaction the two parameters $K_{1,\text{EH}}$ and K_{ESH} were obtained from a fit of eqn. (4) to the $K_1(\text{H}^+)$ results obtained in the pH range 5–3 (Figure 6). This yielded $K_{1,\text{EH}} = 6.4 \text{ mM}$ and $K_{\text{ESH}} = 16.5 \mu\text{M}$ ($pK_a = 4.78$), each in good agreement with the result obtained from Figure 5 ($K_{1,\text{EH}} = 6.3 \text{ mM}$ and Figure 3 ($K_{\text{ESH}} = 18.8 \mu\text{M}$) respectively. Scheme 2 summarizes the results obtained. Three protonation reactions influence the presteady-state kinetics of glucoamylase-catalysed hydrolysis of maltose in the pH range 7–3. First protonization of an enzymic group with $pK_a = 5.7$ in the free enzyme and $pK_a = 6.5$ in the first enzyme–substrate complex makes the K_1 value decrease from 40 to 6.3 mM, but has no influence on k_2 . The second reaction is equally fast, $k_2 = 1640 \text{ s}^{-1}$, of the protonated ESH and the unprotonated ES. There next appears a pH effect, slightly decreasing both K_1 and k_2 , with a pK_a value of 4.5 for the free enzyme and of 4.75 for the enzyme–substrate complex.

Finally, the k_2 value decreases and apparent H^+ saturation of this enzymic group with a pK_a of 2.7 leads to an apparent final value of $k_2 = 0$, whereas this process shows no influence on the K_1 value.

Scheme 2 Reaction scheme showing the pH-dependence of two of the three reaction steps of glucoamylase G1-catalysed hydrolysis of maltose

The dominating reaction(s) at a given pH is indicated. k_c , k_{cat} .

Figure 3 $[\text{H}^+]$ -Dependence in the pH range 7–4.25 of the presteady-state kinetic parameter $k_2(\text{H}^+)$ of glucoamylase G1-catalysed hydrolysis of maltose

As expected from eqn. (3), the difference $[1640 - k_2(\text{H}^+)]$ (in s^{-1}) follows a saturation curve when plotted against the concentration of $[\text{H}^+]$. The curve is that obtained from fitting eqn. (3) to the data with the resulting values of the parameters $k_{2,\text{EH}} = 1090 \text{ s}^{-1}$ and $K_{\text{ESH}} = 18.8 \text{ mM}$.

Figure 4 $[\text{H}^+]$ -Dependence in the pH range 3.74–2.75 of the presteady-state kinetic parameter $k_2(\text{H}^+)$

In this range a second protonization is observed. The curve shown results from fitting of eqn. (3) using the final value of the first protonization 1090 s^{-1} as $k_{2,\text{E}}$.

DISCUSSION

The generally accepted mechanism of hydrolysis catalysed by glucoamylases involves proton transfer to the glycosidic oxygen of the scissile bond from a general acid catalyst, formation of an oxocarbenium ion-like transition state and a nucleophilic attack of water assisted by a general base catalyst [9–12]. The catalysis occurs with inversion of the anomeric configuration in a single

displacement mechanism and the distance between the catalytic acids is $9.2 \text{ \AA}$, as is typical for inverting glycoside hydrolases [11,33]. Studies on mutant glucoamylases have shown further a number of other acid residues that affect the glucoamylase-catalysed reactions. Thus mutation of Asp-55 [34,35] and Glu-180 [19,23,36], respectively, show marked effects on the catalytic rate and on the substrate binding.

Figure 5 $[H^+]$ -Dependence in the pH range 7–5.25 of the presteady-state kinetic parameter $K_1(H^+)$ of glucoamylase G1-catalysed hydrolysis of maltose

The curve shown results from fitting of eqn. (4) to the data.

Figure 6 $[H^+]$ -Dependence in the pH range 5–3.5 of the presteady-state kinetic parameter $K_1(H^+)$ of glucoamylase G1-catalysed hydrolysis of maltose

The curve shown results from fitting of eqn. (4) to the data.

Here the pH-dependencies of the first and second steps of glucoamylase G1-catalysed hydrolysis of maltose have been investigated. The presteady-state kinetic parameters obtained show the participation of three groups ionizing in the pH range 7–2.5 (Scheme 2). The first one to be protonated when the H^+ concentration increases shows a pK_a of 5.7 for the free enzyme, E, and a pK_a of 6.5 for the enzyme–substrate complex, ES_1 . Since it is found that k_2 is not influenced, and that k_{-2} is approximately the same, the pK_a value of this group is also ≈ 6.5 of the Michaelis complex, ES_2 , and is thus the one that shows its effect on k_{cat} . This is in excellent agreement with the steady-state kinetic results, where a pK_a value of 6.4 was obtained from the dependence of k_{cat} on H^+ at 8 °C (results not shown). The group with $pK_a \approx 6.5$ is thus the one defined as the general acid catalyst of glucoamylase and identified as Glu-179. Protonation/deprotonation of this group has no effect on the rate of the second reaction step of model 1; k_2 does not change. We are therefore able to conclude that a conformational change and not hydrolysis of the substrate is occurring in the second reaction

step. The rate-determining step of glucoamylase-catalysed reactions with the rate constant k_{cat} , which follows the second step and is dependent on a protonated form of Glu-179, must then be the hydrolysis step. It is impossible that the hydrolysis step can be independent of the presence or absence of a protonated catalytic acid but totally govern the release of products, as has been claimed recently [24–26].

The other groups of $pK_a \approx 4.5$ and ≈ 2.75 found here to affect the presteady-state kinetics of glucoamylase are less easy to identify. Glu-400 immediately is a candidate at least for the group with $pK_a \approx 2.75$, whereas the observed effects are too small and relate to a weaker acid of the group of $pK_a \approx 4.5$. In our previous studies of the presteady-state kinetics of glucoamylase and mutants thereof [8a], the second step of the reaction, which, as can now be said with confidence, represents a conformational change resulting in the optimal enzyme–substrate configuration for hydrolysis, was shown to depend on side chains on the β -flank of the active site (Figure 1). Glu-180 plays an important role here, the pattern of hydrogen bonds between Arg-305, Asp-309, Tyr-306 and Glu-180 is disturbed when Glu-180 is mutated to Gln [8a] and protonation of Glu-180 most probably would result in the same effect with marked reduction of k_2 . Asp-309 of course may be another candidate. Each of these may be the group with a pK_a of 2.75 (Scheme 2), which strongly affects the rate of the second reaction step, but not K_1 . Protonation of this group actually seems to hinder the formation of the normal Michaelis complex to such an extent that this and not the protonation of the catalytic base would govern the decrease of the steady-state rates at very low pH, if the enzyme was stable here.

The presteady-state kinetic parameters are only slightly perturbed by the group of $pK_a \approx 4.5$, and this group does not affect the steady-state kinetics according to previous pH studies [1,13–16]. So this may be any acid group that is not of great importance, perhaps influencing the reaction slightly at a distance from the active site. Also, since the pK_a value in itself makes it possible that the effects stem from the buffer, an acetate ion or an acetic acid molecule may interact with the enzyme and lead to small kinetic effects.

A rate-determining hydrolysis step is supported in model 1:

The claim that product dissociation and not hydrolysis should be the rate-determining step governed by k_{cat} [25,26], seems highly unlikely in the light of the known pH-dependency of k_{cat} [1,14–16], the weak binding of the product glucose [2] and results showing the slower, but nevertheless fast, second reaction steps of a number of mutants [8a,17–19]. The present study of the pH-dependence of the presteady-state kinetic parameters of the interaction of wild-type glucoamylase G1 and maltose has shown further that the k_2 value slightly increases in the range pH 5–7, and does not decrease with the pK_a of the catalytic acid, as would be the case if this step was the actual hydrolysis step.

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