Kinetic studies of the polygalacturonase enzyme from *Colletotrichum lindemuthianum*

Gabriel WAKSMAN,[†] Geoffrey TURNER and Adrian R. WALMSLEY*

The Krebs Institute, Department of Molecular Biology and Biotechnology, The University of Sheffield, P.O. Box 594, Firth Court, Western Bank, Sheffield S10 2UH, U.K.

The intrinsic protein fluorescence of the polygalacturonase from *Colletotrichum lindemuthianum* was exploited in stopped-flow experiments aimed at elucidating the kinetic mechanism for this enzyme. Binding of the polymeric substrate polygalacturonic acid (PGA) essentially produced a triphasic fluorescence profile. There was an initial rapid quench in fluorescence, consistent with the rapid formation of the enzyme–substrate complex, with an equilibrium constant of about 8×10^{-4} % (w/v) PGA (about 0.27 μ M). There then followed a near-constant fluorescence phase, attributable to turnover of the enzyme–substrate complex as a steady-state intermediate. As the concentration of the steady-state intermediate became depleted, towards the end of the reaction, there was a partial return of the fluorescence intensity. This phase is attributed to a final, single turnover of the enzyme at the end of the reaction. The fluorescence intensity does not return to its original level due to product remaining bound at the end of the reaction.

INTRODUCTION

Saprophytic and plant pathogenic fungi and bacteria produce an array of enzymes capable of degrading the complex polysaccharides of the plant cell wall, including pectin, the main constituent of the middle lamella of higher plant cells [1]. Pectin is a heteropolysaccharide consisting of a linear polymer of α 1 $\rightarrow$ 4-bond D-galacturonic acid residues or polygalacturonic acid (PGA), which is partially esterified. The microbial degradation of pectin is a complex process, involving several enzymes. One of these is polygalacturonase (PG) (EC 3.2.1.15), which is able to cleave hydrolytically the α 1 $\rightarrow$ 4-bond of esterified (pectin) or non-esterified (pectic acid) polygalacturonic acid [2,3].

PGs have been purified and characterized from a number of organisms. Although they can show extensive variation in their physical and chemical properties, they also exhibit some common structural and enzymological features [4,5]. For example, an amino acid domain which is common to several different PGs found in ascomycete fungi has been reported [4]. Few enzymological studies have been performed upon these enzymes. However, there have been several reports that the hydrolysis rate of oligogalacturonic acids (OGAs) catalysed by PGs from different organisms decreases upon shortening of the substrate chain [6,7], a process that will ultimately lead to substrate/product inhibition of the reaction. Several PGs, from different organisms, have been found to be inhibited by high-molecular-weight inhibitors produced by plants [8,9].

Further structural and enzymological studies, using material-consuming techniques like X-ray crystallography, n.m.r. and protein fluorescence stopped-flow spectroscopy, have hitherto been considerably hampered either by the relatively small amount of enzyme synthesized by PG-producing organisms, or by the difficulties associated with purifying single components of an often multi-isoform complex.

Recently we have established protocols for the purification of large amounts of single PG isoforms from three fungi; *Colletotrichum lindemuthianum*, *Aspergillus niger*, and *Sclerotinia sclerotiorum* [4,10]. While the PG systems produced by either *A. niger* or *S. sclerotiorum* consisted of several isoenzymes with low

to neutral pI, the PG system produced by *C. lindemuthianum* consisted of a single component with a high pI. These features of the *C. lindemuthianum* PG system have enabled us to set up a simple and low-cost purification protocol, which is far more cost-effective than that proposed for the purification of the *A. niger* and *S. sclerotiorum* PG isoforms. The rapid processing of a large volume of culture filtrate through an SP zeta-prep disk has made it possible for us to purify protein in quantities well above 0.1 g. Furthermore, several transformation systems have been reported for this organism [11,12], allowing the transfer of homologous or heterologous genes to be undertaken, and making the *C. lindemuthianum* PG system amenable to genetic manipulation. More recently a PG-encoding gene has been isolated from *A. niger* [13], which should be invaluable as a probe for isolating the *C. lindemuthianum* PG gene.

These features of the *C. lindemuthianum* PG system make it an attractive source of the enzyme for studies aimed at elucidating the structure and function of this class of enzymes, about which there is currently little known. Towards this end we are currently attempting both to crystallize the enzyme and to elucidate its mechanism of action. With the latter goal in mind, we have exploited the observed changes in the intrinsic protein fluorescence of the enzyme in transient kinetic studies aimed at elucidating the elementary steps of the reaction mechanism. In the present study we demonstrate a quench in tryptophan fluorescence of the enzyme upon binding with PGA, which can be used to provide information upon transient intermediates which occur along the reaction pathway. These studies, and future studies with OGAs of varying chain length, will be an important step towards an understanding of the mechanism of action of PGs.

MATERIALS AND METHODS

Chemicals

PGA was obtained from Serva. The polymer was reported to have an average chain length of 170 residues and a molecular mass of 30 kDa.

Abbreviations used: PGA, polygalacturonic acid; OGA, oligogalacturonic acid; GA, galacturonic acid; PG, polygalacturonase (EC 3.2.1.15).

* To whom correspondence should be addressed.

[†] Present address: Laboratory of Molecular Biophysics, The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A.

Isolates and cultures

C. lindemuthianum (Sacc. et Mag.) Bri. et Cav. race I57 (IMI 334960) was a gift of J. A. Bailey (AFRC Institute of Arable Crops Research, Long Ashton Research Station, Bristol, U.K.). The isolate of *C. lindemuthianum* was maintained and spore suspensions were produced as previously described [14]. For enzyme production, the fungus was grown as described by Keon & Waksman [4].

Purification of the PG enzyme from *C. lindemuthianum*

The purification of the PG enzyme from *C. lindemuthianum* is as described by Keon & Waksman [4]. Purity of the enzyme was checked using SDS/PAGE and analytical isoelectric focusing as described in [4]. The protein concentration was determined from the 280 nm absorbance, using a specific absorption coefficient of $0.925 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$. This coefficient was determined by measuring the absorbance at 280 nm of a sample of known weight, determined from the amino acid composition of the sample using a Waters Pico-tag system (at the University of Sheffield). A molecular mass of 38 kDa was used for calculation of the enzyme active-site concentration [4].

Steady-state enzymic assays

Enzymic activity was determined using the Nelson-Somogyi method [15]. Reaction mixtures consisted of 1 ml of PGA (at the required concentration) in 50 mM-acetate, buffered to the required pH with glacial acetic acid, and $2.5 \mu\text{l}$ of $0.06 \mu\text{M}$ -PG. Incubations were performed at 20°C for 5 min.

Spectroscopic equipment

Rapid reactions were followed using an Applied-Photophysics (London, U.K.) spectrofluorimeter. Tryptophan fluorescence was excited at 297 nm, selected by focusing a 150 W xenon arc lamp onto an $f/3.4$ monochromator, and the emitted light was selected with a WG335 Schott filter positioned in front of the observation photo-multiplier tube. Data were captured on an Archimedes 420/1 microcomputer, and analysed by non-linear regression. At least three stopped-flow traces were averaged prior to non-linear regression analysis.

Spectroscopic assays

All reactions were monitored in 50 mM-sodium acetate, buffered to pH 4.2 with glacial acetic acid, at 20°C . The quoted concentrations in stopped-flow experiments refer to those in the syringes, unless stated otherwise. In all the experiments reported here, equal volumes ($50 \mu\text{l}$) of enzyme and PGA solutions were mixed in the stopped-flow apparatus. As a routine check of the behaviour of different enzyme preparations, often from different fungal batches, the fluorescence profiles for turnover of 0.25% PGA by $6 \mu\text{M}$ enzyme were compared. The activity of different preparations was also checked for consistency by using the reducing-group assay described above.

Production of PGA hydrolysate

PGA 0.5% was hydrolysed overnight with $1 \mu\text{l}$ of $0.06 \mu\text{M}$ -PG/ml, at 20°C . PG was then heat-inactivated and the hydrolysate was filtered through a $0.22 \mu\text{m}$ Amicon filter.

RESULTS

The binding and subsequent turnover of a limited excess of PGA by PG, at pH 4.2, is characterized by a multiphasic tryptophan fluorescence profile, with at least three clearly defined phases (Fig. 1a). First, there is rapid quench in fluorescence; followed by a steady-state phase, during which the fluorescence quenching remains constant; and finally a decay in the fluores-

cence quenching to a protein fluorescence level below that of the ligand-free protein.

PGA binding

The first phase, which is presumably attributable to the reversible binding of PGA to the enzyme, was too fast to be measured by stopped-flow spectroscopy. However, while this initial fluorescence quench could not be time-resolved, its amplitude was found to increase hyperbolically with the substrate concentration, allowing assessment of the steady-state parameters for PGA binding. Fitting of the data to a simple hyperbola indicated a dissociation constant for the substrate-enzyme complex of about $8.0 \times 10^{-4} \%$ (w/v) PGA, with a maximal quench of about 12.5% (Fig. 2). The K_d value would correspond to a molar value of about $0.27 \mu\text{M}$, indicating that the PGA is bound with high affinity.

Fig. 1. PGA turnover by PG

Illustrative stopped-flow records showing the changes in protein fluorescence during a limited turnover of PG. In each case the upper trace represents the baseline fluorescence when $12 \mu\text{M}$ -PG was mixed with sodium acetate (50 mM) buffered to pH 4.2. The lower trace shows the protein-fluorescence changes occurring when $12 \mu\text{M}$ -PG was mixed with (a) 0.05% PGA and (b) 0.5% PGA. Assays were performed in 50 mM-sodium acetate buffer (pH 4.2) at 20°C . Note the difference in the time-bases of the two sets of traces. The boxed area indicates the slow decrease in protein fluorescence which is analysed further in Fig. 3. One vertical division represents a 2.5% fluorescence change.

Fig. 2. Initial quench in protein fluorescence as a function of [PGA]

The initial quench in protein fluorescence due to PGA binding is plotted against [PGA] in the stopped-flow mixing chamber. The best-fit hyperbola yielded an apparent dissociation constant of 8.0×10^{-4} % PGA ($0.27 \mu\text{M}$) and a maximum quench of 12.5% for PG saturated with PGA.

Fig. 3. PGA binding to PG

The stopped-flow records show the slow phase of protein quenching on binding with PGA (boxed area in Fig. 1). The reactions were initiated by mixing of $12 \mu\text{M}$ -PG with (a) 0.5% PGA and (b) 0.25% PGA. Assays were performed in 50 mM-sodium acetate buffer (pH 4.2) at 20 °C. A single exponential fit is superimposed on each trace, yielding rate constants of (a) 2.21 s^{-1} and (b) 3.35 s^{-1} . Note the difference in time-bases of the two records. One vertical division represents a 0.5% fluorescence change.

For relatively high substrate concentrations, more than 10-fold the apparent K_d value, there was a decrease in the quenching, with a second, slow phase emerging during the first 2 s of the reaction (Fig. 1b). The slow decrease in the fluorescence of the protein which occurs during this phase of the reaction is illustrated in Fig. 3. Unfortunately, a rigorous analysis of the substrate concentration dependence of this second phase was not

Fig. 4. PGA turnover in the steady state as a function of [PGA]

The apparent steady-state rate of PGA turnover was calculated from the duration of the constant-fluorescence phase monitored in limited turnovers of PGA. The rates were calculated as the percentage of PGA turned over during this period. Fitting of a hyperbola to the initial data (amplified in the inset), before inhibition became apparent, yielded a K_m value of 7.8×10^{-4} % ($0.26 \mu\text{M}$) PGA and a V_{max} value of 1.97% PGA degraded/min per μM of PG. Fitting of a decreasing hyperbolic function to the inhibition data indicated that the rate approaches a lower limit of 312 s^{-1} at saturating [PGA].

possible for several reasons. First, it was of very small amplitude, being around 10% of the total protein fluorescence quench at the highest PGA concentration used, and decreased with the substrate concentration. Secondly, the duration of the steady state was progressively shortened with decreasing substrate concentration, making observation of this phase at low substrate concentrations difficult. However, it was apparent that the rate of this phase decreased with increasing substrate concentration, so that for 0.25% PGA this phase had a rate of about 2.21 s^{-1} , while reducing the [PGA] to 0.125% caused the rate of this phase to increase to about 3.35 s^{-1} (Fig. 3).

Steady-state turnover of PGA

The steady-state fluorescence phase presumably provides a measure of the rate of enzyme turnover in the steady state, since this phase can be proportionally prolonged by either increasing the substrate concentration or by reducing the enzyme concentration (see Figs. 1 and 7). Taking the duration of this phase as the time required for the enzyme to degrade a specified PGA concentration, the rate of polymer degradation can accordingly be calculated. Although such measurements do not provide an accurate measure of the steady-state rate, the data clearly demonstrate that the rate is saturable with respect to the substrate concentration, with inhibition becoming apparent at higher substrate concentrations (Fig. 4). The data indicate a K_m -value of 7.78×10^{-4} % and a V_{max} value of about 0.39% PGA degraded per second. This V_{max} -value corresponds to a polymer turnover number for the enzyme of 10.9 s^{-1} . This information can be used to give an upper limit for the steady-state rate of bond cleavage. Assuming that there are 170 galacturonic acid (GA) residues per polymer, all of which are cleaved, then this would equate with a cleavage rate of about 1.85 mM/s per μM of enzyme, or an apparent k_{cat} value of about 1850 s^{-1} . However, these figures are dependent upon the proportion of available bonds cleaved. At high substrate concentrations, inhibition of the turnover rate becomes clear. Fitting of these data to a decreasing hyperbolic function indicated that the rate approaches a value of 312 s^{-1} at

Fig. 5. Steady-state rate of PGA cleavage as a function of [PGA]

Rates of PGA cleavage were determined using the Nelson–Somogyi reducing-group assay [15]. Reactions were initiated by the addition of 2.5 μ l of PG (A_{280} 0.01) to 1 ml of PGA, at the required concentration in 50 mM-sodium acetate buffer (pH 4.2; 20 °C). Reactions were terminated after 5 min by placing the assay tubes in boiling water. The rates were calculated as μ M of GA equivalents released/s per μ M of PG, which simply equates with the turnover number (s^{-1}).

Fig. 6. Steady-state intermediate decay rate

The rate constants for the return in protein fluorescence observed at the end of a limited PGA turnover is plotted as a function of the initial [PGA]. Rates were determined by fitting of the latter half of each fluorescence trace to a single exponential function. The best-fit hyperbola for the data yielded a half-saturation constant of 2.98×10^{-3} % PGA and a maximal rate of $300 s^{-1}$.

saturation [PGA], with a half-saturation constant of about 0.23 % PGA (76.7 μ M).

In order to confirm that the nearly constant fluorescence phase was reflecting enzymic turnover in the steady state, a reducing-group assay was used to measure rates of PGA hydrolysis. In one experiment, product formation was monitored under identical conditions to those prevailing in the stopped-flow experiments (e.g., 12 μ M enzyme was mixed with 0.5 % PGA) (results not shown). Under such conditions we found product formation was rapid, with more than 95 % completion within 2 s, a state in which 55 % of the available bonds had been cleaved. These data establish that the constant-fluorescence phase is reflecting turn-

over of the enzyme in the steady state, with both procedures giving a rate of about $580 s^{-1}$. Owing to the apparent substrate inhibition at this [PGA] this rate is less than k_{cat} , which we can now correct for the percentage of bonds cleaved, to give a value of $1018 s^{-1}$. The fact that the reaction had essentially gone to completion within the apparent steady-state period would be consistent with either the enzyme–substrate or the enzyme–product(s) complex as the steady-state intermediate, which is clearly shown to accumulate during the steady-state/constant-fluorescence phase. The reducing-group assay was also used in an attempt to monitor initial rates of product formation, with the aim of determining the steady-state parameters, which could then be compared with those obtained from fluorescence studies. However, our experiments pinpointed a potential complication in making such measurements. For low substrate concentrations the reaction progress curves were non-linear, with apparent enzyme activation occurring. Upon consideration of the catalytic reaction, this behaviour is not unexpected. Initially the enzyme can cleave the polymeric substrate to produce two smaller polymeric fragments, which can act as substrates for further turnovers of the enzyme. In consequence, the substrate concentration will increase with each turnover of the enzyme. For low substrate concentrations, well below K_m , the increase in the apparent rate as the reaction proceeds will be most dramatic. This apparent activation will proceed until substrate fragments are produced which are either turned over more slowly or are resistant to further cleavage. This behaviour has also been noted in an early study, which showed that pectic acid cleavage by the yeast *Saccharomyces fragilis* was biphasic [7]. There was an initial rapid phase of product formation, followed by a slower steady-state phase. The latter phase parallels an initially linear rate of GA production. Furthermore, the affinity for the increasingly smaller OGAs produced may progressively decrease. This would tend to give a higher K_m value for the overall reaction than for the initial rate of PGA cleavage. In view of the difficulties associated with making initial-rate measurements, we have merely monitored product formation over a fixed time interval, which was sufficient to allow less than 10 % PGA cleavage at the higher concentrations employed. Even so, these rates cannot be considered truly initial, with only 55 % of the available bonds being cleaved by the end of the reaction. Unfortunately, such an analysis does not allow an accurate determination of K_m to be made, as illustrated by the non-hyperbolic [PGA] dependence of the steady-state rate, as shown in Fig. 5. This plot is characterized by an almost linear increase in rate at low substrate concentrations, followed by a rapid levelling off of the rate at high substrate concentrations. Elevating the [PGA] further led to substrate inhibition of the reaction (results not shown).

Although an accurate measure of the steady-state parameters cannot be obtained, it is reasonable to conclude from the data that K_m is about 1.5×10^{-3} % PGA and k_{cat} $1200 s^{-1}$. These figures are reasonably comparable with those we have obtained from fluorescence studies, especially when one considers that in one procedure only the early part of the reaction was monitored (activity measurements) and in the other effectively the whole time-course of the reaction was monitored (fluorescence measurements). Indeed, it may be because the whole time-course of the reaction was investigated in fluorescence studies that apparent hyperbolic kinetics were found. One would expect the rate to be an average of the early part of the reaction, where the substrate concentration was increasing, and the latter part of the reaction, where the substrate concentration was decreasing.

PG single-turnovers

Towards the end of the reaction, there is a partial return of the fluorescence intensity of the protein (Fig. 1; steady-state fluores-

Fig. 7. PGA turnover by PG

The stopped-flow records show the protein fluorescence changes associated with the turnover of 0.005% PGA by (a) 6 μM - and (b) 18 μM -PG. Assays were carried out in 50 mM-sodium acetate (pH 4.2; 20 °C). Note the 3-fold increase in the steady-state phase, while the single-exponential fits superimposed upon the exponential phase of the traces yield similar rate constants of (a) 124 s^{-1} and (b) 119 s^{-1} , indicative of a single turnover. One vertical division represents a 2.5% fluorescence change.

Fig. 8. Quench in protein fluorescence associated with product binding

The resulting quench in protein fluorescence following PGA turnover by PG is shown as a function of the initial [PGA]. The best-fit hyperbola indicated an equilibrium constant of 2.5×10^{-2} % (PGA equivalents) and a maximal quench of about 10%.

cence results not shown). This decay in fluorescence quenching presumably represents the decrease in the concentration of the steady-state intermediate. When the latter half of each intermediate decay curve was analysed, the data could be fitted to a single exponential. The rate of this phase was found to decrease hyperbolically with increasing substrate concentration (Fig. 6). Fitting of the data to a decreasing hyperbolic function gave a half-saturation constant of 2.98×10^{-3} % and a maximal rate of about 300 s^{-1} . To ascertain whether or not this behaviour was due to an increasing number of enzyme turnovers being required

to deplete the concentration of the steady-state intermediate as the [PGA] was increased, the effect of increasing the concentration of PG was investigated. Fig. 7 illustrates the effect of a 3-fold increase in the enzyme concentration on the turnover of 0.005% PGA. The steady-state period was enhanced about 3-fold, while the decay phase was hardly affected. This behaviour was subsequently found to be consistent over the whole concentration range studied (results not shown). This strongly suggests that most of the PGA is being hydrolysed during the steady-state period, with the enzyme undergoing a single turnover at the end of the reaction. However, the observed decrease in rate of this phase would not be consistent with a first-order decay in the concentration of the steady-state intermediate towards the end of the reaction, but might be explained if all the mechanistic steps subsequent to formation of the steady-state intermediate were readily reversible, or, alternatively, that there was product inhibition of the reaction. Irrespective of the cause of this behaviour, this finding has enabled us to determine the rate constant for a single turnover of the enzyme by back-extrapolation to zero substrate concentration. This yielded a single turnover rate of about 300 s^{-1} . The fast rate and small amplitude of the signal change involved in a single turnover of the enzyme make direct measurements difficult.

Product binding

The protein fluorescence does not return to its original level at the end of the reaction, indicating that the final reaction product(s) remain bound to the enzyme. Indeed, for PGA concentrations as high as 0.25% there is only a 30% return in fluorescence intensity at the end of the reaction, implying that the reaction products are bound with relatively high affinity. Consistent with this interpretation, the amplitude of the decay phase progressively diminishes with increasing substrate concentration, as would be expected with increasing amounts of product remaining bound at the end of the reaction. Product binding was demonstrated directly by mixing of 0.25% hydrolysed PGA with 12 μM enzyme. This resulted in about a 6% quench in the protein's fluorescence, which occurred within the dead-time of the stopped-flow experiment; thereafter the protein fluorescence remained constant. These fluorescence changes cannot be attributed to GA binding at the end of the reaction, since GA was found neither to inhibit the enzyme nor to quench the protein fluorescence (results not shown). These results are indicative of the rapid formation of a high-affinity enzyme-OGA complex at the end of the reaction. A plot of the amplitude of the fluorescence quench associated with product binding, as a function of the initial [PGA] added (and hence the [product] generated), is shown in Fig. 8. Analysis of these data in terms of a hyperbola indicated a binding constant of about 2.5×10^{-2} % (PGA equivalents) for the product. These data give a lower limit for the product dissociation constant of about 8 μM , but it will clearly be greater due to the increase in concentration of product OGAs as the reaction proceeds. However, the affinity of the enzyme for the product OGA must be less than for the substrate PGA, since a 100-fold excess of hydrolysed PGA (product) did not significantly decrease the rate of 0.005% PGA binding or steady-state turnover, irrespective of whether or not the enzyme was pre-incubated with the hydrolysed PGA (results not shown).

DISCUSSION

In this paper we have established that the binding of the substrate, PGA, to the PG from *C. lindemuthianum* causes a quench in the protein fluorescence. The kinetics of the fluorescence changes, monitored by stopped-flow spectroscopy, during

a limited turnover are informative of several elementary steps in the reaction mechanism.

A minimal kinetic scheme around which to interpret these results is presented in equation (1)

where P_1 and P_2 are the hydrolysis products, which may be either GA or fragments of the cleaved PGA molecule, E is the enzyme, S is substrate and ES , EP_1P_2 and EP_2 are complexes. Consider the kinetic data presented in this paper in terms of this model. There was an initial rapid quench in fluorescence, too fast to measure by stopped-flow techniques, indicating that formation of the enzyme-substrate complex (ES) is rapid, with a rate constant greater than the lower limit set by the dead-time of the stopped-flow apparatus (approx. 340 s^{-1} with a dead-time of about 2 ms). Steady-state measurements of PGA cleavage have revealed that the hydrolysis step is also rapid, with a rate of at least 1000 s^{-1} . Again, this would be too fast to measure by stopped-flow techniques. In consequence, no clear decision on the identity of the intermediate which accumulates during the steady-state phase would seem possible. However, calculation of the specificity constant (k_{cat}/K_m), to yield a value of $3.77 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, would suggest that the rate-limiting step at low substrate concentrations is the association of substrate and enzyme. Although this value was calculated on a PGA molar basis, even on a GA molar basis the second-order rate constant would be $2.22 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. This value is still sufficiently high, in relation to the upper limit for a diffusion-controlled encounter of $10^9\text{--}10^{11} \text{ M}^{-1} \text{ s}^{-1}$, so as to suggest that this second-order rate constant is a measure of the enzyme-substrate association rate constant. Such a finding leads us to suggest that the enzyme is obeying Briggs-Haldane kinetics, in that the cleavage step is faster than the dissociation rate constant for the enzyme-substrate complex. Indeed, use of the calculated values for K_m , k_1 and k_2 (assumed to be equivalent to k_{cat}) and the relationship $K_m = (k_{-1} + k_2)/k_1$ would indicate that k_{-1} is relatively slow compared with k_2 , being less than 1 s^{-1} even when calculated on a GA molar basis. In accord with these conclusions, the apparent K_s value was found to be very similar to that of K_m . This is not surprising, since substrate binding is essentially irreversible, due to the slow dissociation and rapid hydrolysis steps. Under such circumstances K_s would be expected to give a measure of k_2/k_1 , and the observed steady-state intermediate would largely be the enzyme-substrate complex. For such a model, dissociation of the two products would have to be rapid relative to the cleavage step. This requirement of the model is supported by the finding that preincubating the enzyme with 0.25% hydrolysed PGA (product) did not appreciably affect the binding and turnover of 0.005% PGA. This would suggest either that the binding of the product to the enzyme is a rapid equilibrium process or that little of the enzyme-product complex is formed due to it having a high K_d value. The latter suggestion can be rejected since we have shown that the K_d value for the enzyme-product complex is 0.025% (w/v PGA equivalents). Since GA has been shown to be non-inhibitory for PGA turnover, these findings would point towards a rapid-equilibrium, random-order release of products.

We have shown in this study that GA has a low affinity for the enzyme, indicating that the product remaining bound to the enzyme is an OGA. Consistent with this interpretation, previous studies have shown that PGA is broken down by PG to tri-, di- and mono-galacturonic acids, while only trigalacturonic acid is

inhibitory for the reaction [6,7]. Our figure of 55% hydrolysis would be consistent with the equimolar production of these three products. T.l.c. analysis has established that only three products are formed in the final reaction mixture, one of which is GA (G. Waksman, unpublished work). It is tempting to suggest that trigalacturonic acid is the product remaining bound at the end of the reaction.

At relatively high [PGA], a slow transient was observed prior to the steady-state phase (see Fig. 3). This transient cannot be attributed to formation of an in-line intermediate of the mechanism, such as the ES or EP_1P_2 complexes, since its rate is at least two orders of magnitude slower than k_{cat} . This behaviour might be attributable to a slow equilibration of the enzyme between conformations, which differ in their activity. The fact that the rate for this transient decreases with increasing [PGA] would suggest that the enzyme is in equilibrium between these conformations prior to substrate binding, which perturbs the equilibrium. The apparent substrate inhibition, noted at high [PGA], might also be reconciled with this mechanism. If one conformation is characterized by a low affinity and slow turnover rate relative to the other conformation, then a high [PGA] would tend to pull the enzyme into the less active conformational form, leading to substrate inhibition. Indeed, the steady-state reaction rate drops to about 300 s^{-1} , which is comparable to the single-turnover rate, but about 3–4-fold lower than the maximal steady-state rate. This behaviour would suggest that most of the PGA is hydrolysed by the more active conformation, with the less active conformation effectively undergoing a single turnover at the end of the reaction at a slower rate than k_{cat} .

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