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Pre-steady-state kinetic studies of rat kidney γ-glutamyl transpeptidase confirm its ping-pong mechanism†

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ABSTRACT: The enzyme γ-glutamyl transpeptidase (GGT) is implicated in cellular detoxification, the biosynthesis of leukotrienes and control of the physiological concentration of glutathione. It also plays important roles in Parkinson's disease, diabetes, apoptosis inhibition and cancer drug resistance. It catalyses the breakdown of its *in vivo* substrate, glutathione, by cleaving the amide bond between the γ-glutamyl and the cysteinylglycine moieties. Threonine is proposed to act as the nucleophile of GGT in the formation of the γ-glutamyl acyl enzyme intermediate during the acylation step. The γ-glutamyl moiety is then transferred to a primary amine acceptor substrate (an amino acid or dipeptide) or to a water molecule, to form a compound containing a new isopeptide bond or glutamate in the transamidation or hydrolysis reactions, respectively. In spite of the importance of the role of GGT in human physiology, there is a lack of information about the mechanisms of its catalytic reactions, and in particular the nature of the intermediate formed during the acylation step. In order to gain insight into the formation of the acyl enzyme intermediate, different D-γ-glutamylanilides substituted in the *para* position with electron-withdrawing and electron-donating groups were used as donor substrates under conditions where water served as acceptor substrate. A Hammett plot with a slope of zero was obtained for the steady-state hydrolysis reaction for which deacylation is the rate-limiting step. To confirm the ping-pong mechanism, pre-steady-state kinetics of this reaction were then performed with the donor substrate D-γ-glutamyl-*p*-nitroanilide, which liberates the chromophore *p*-nitroaniline. Experiments using a stopped- flow spectrometer and a rapid mix–quench apparatus gave biphasic traces with a burst up to ∼65 ms, the amplitude of which corresponds well with the concentration of the enzyme. These burst kinetics were also observed in the presence of L-methionine at concentrations ∼15-fold below its K_M value, where deacylation would still be rate limiting. These observations are consistent with the formation of an intermediate during the rapid acylation step and support the modified ping-pong mechanism proposed for GGT- mediated hydrolysis and aminolysis. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: enzyme mechanism; γ-glutamyl transpeptidase; pre-steady-state kinetics; hydrolysis; transpeptidation; Hammett plot

INTRODUCTION

γ-Glutamyl transpeptidase (GGT) (EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme bound to the external surface of cells in mammals,[1,2] bacteria[3] and plants.[4] It is found mainly in kidney, but is also present in brain, pancreas and liver.[5,6] This enzyme has been implicated in many physiological processes in humans, including cellular detoxification through the formation of mercapturic acids,[7] biosynthesis of leukotrienes D_4[8] and regulation of glutathione (γ-glutamylcysteinylglycine) concentration through the γ-glutamyl cycle.[9] It has also been found to be implicated in many diseases, such as Parkinson's disease,[5] diabetes[10] and apoptosis inhibition.[11] Despite its broad biological importance in mammals, its complete mechanism of action and the nature of the amino acids implicated in the active site are not yet known.

GGT binds glutathione as its *in vivo* donor substrate and cleaves the amide bond between the γ-glutamyl and the cysteinyl moieties in an acylation step. The γ-glutamyl moiety can then be transferred to a broad range of amino acids and dipeptides of L-configuration only (in a transpeptidation reaction) or to a water molecule (in a hydrolysis reaction) to give a new γ-glutamyl peptide or glutamate, respectively. A modified ping-pong mechanism has been proposed for GGT, as represented in Scheme 1.[1,2,12] In order to study the acylation and deacylation steps separately, the donor and acceptor substrates must be chosen carefully to determine which step will be rate limiting. Furthermore, a donor substrate can also act as an acceptor substrate, in a competing autotranspeptidation reaction, if it is of L-configuration.

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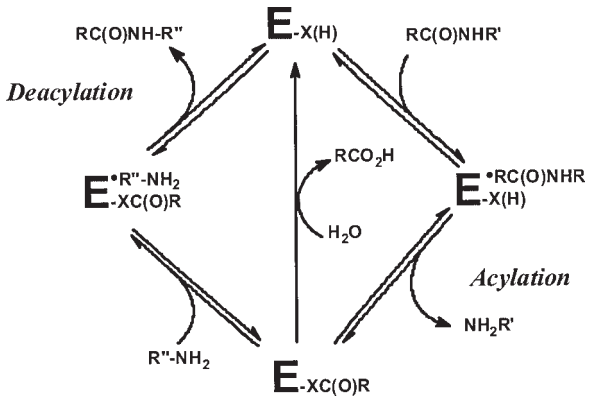

Scheme 1

Thus, in order to study the hydrolysis reaction separately, donor substrates with D-configuration are used in the absence of primary amine acceptor substrates. One such donor substrate widely used in activity assays and mechanistic studies is D-γ-glutamyl-*p*-nitroanilide, shown in Fig. 1 ($X = NO_2$), which is known to display similar kinetic properties to glutathione.[2]

The active-site amino acid residues implicated in the catalytic reactions of GGT are not well known. For example, the nucleophile has not been identified for all mammalian GGTs. Ser-451 and -452 have been suggested by site-directed mutagenesis to act as a nucleophile in human GGT.[13] In *E. coli* GGT, the N-terminal amino acid of the small subunit, Thr-391, has been identified by enzymatic digestion and mass spectrometry to be the nucleophile.[3] This residue is conserved in all GGTs, leading the authors to propose that GGT is a member of the Ntn-hydrolase family. Other characteristics of enzymes belonging to this family include their activation through autoprocessing of enzyme precursors[14] and the presence of two antiparallel β-pleated sheets, both of which are present for bacterial GGT.[3,15] We have proposed that an amino acid must act as a general acid catalyst for the protonation of the leaving group during the acylation step. In our previous studies of GGT, we used a series of L-γ-glutamylanilides bearing electron-withdrawing or electron-donating groups in the *para* position, whereby the strength of the cleaved amide bond varies according to the substituent.[16] The resulting Hammett plot of our observed kinetic data, together with a pH–rate profile, isotope effect studies and Eyring and van't Hoff plots, allowed us to identify a Lys or His residue as bearing the ammonium group playing this role. However, the absence of a precise crystal structure of any GGT prevents the unambiguous identification of the amino acids implicated in catalysis.

Although several different lines of evidence from GGT mechanistic studies are consistent with the ping-pong catalytic cycle shown in Scheme 1, some authors have proposed that a sequential mechanism may also be consistent with certain kinetic data.[17] The formation of an acyl enzyme intermediate, which is highly probable for enzyme-mediated acyl transfer reactions (see, for example, Ref. 17 and references cited therein), would be direct support of a ping-pong mechanism. In order to prove the presence of this enzyme form, Elce and Broxmeyer incubated GGT in the presence of doubly radiolabeled glutathione and observed that more radioactive [^{14}C]-γ-glutamyl enzyme was detected than radioactive [^{3}H]glycine bound on the enzyme.[18,19] They also incubated rat kidney GGT with *N*-acetylimidazole, which can react with free amino groups. After this incubation, a form of GGT was obtained that was capable of binding [^{14}C]glutathione, but had a limited capacity of catalysing its hydrolysis. Unfortunately, it appears that hydrolysis was fast enough to prevent the accumulation of a sufficient quantity of acyl enzyme intermediate for thorough characterization. However, upon consideration of the stability of the new bond formed with the modified enzyme, they concluded that an amide bond had been formed. When Smith and Meister performed the same kind of experiment with *N*-acetylimidazole, they obtained, after 20 s of incubation, a modified acyl enzyme that was no longer able to liberate *p*-nitroaniline from the donor substrate L-γ-glutamyl-*p*-nitroanilide.[20] However, in both of these studies, an inhibitor was used that was proposed to react with GGT distant from the active site, exposing previously buried amino acids. For example, after treatment with *N*-acetylimidazole, some residues were exposed that could react with iodoacetamide, inhibiting binding in the donor substrate binding site.[19,20] Clearly, the discussion of the identification of the acyl enzyme would be aided by the direct detection of transient intermediates using analogues of glutathione as donor substrates that would lead to the formation of an acyl enzyme more closely resembling the one formed physiologically.

Here we present a Hammett plot constructed using kinetic results from the GGT- mediated hydrolysis reaction of a series of γ-glutamylanilides containing amide bonds of different strength. These results suggest that for this process, acylation is not the rate-limiting step. Several different pre-steady-state kinetic experiments were then performed in order to observe the formation of an acyl enzyme intermediate on the millisecond scale and to confirm the nature of the rate-limiting step. The direct observation of a burst due to the rapid liberation of the product at initial reaction times is representative of

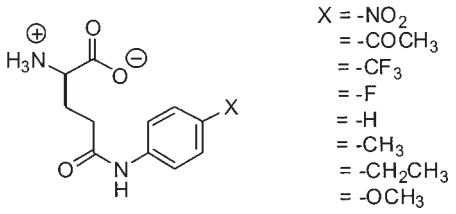

Figure 1. Synthetic γ-glutamyl donor substrates used in this study

the formation of such an acyl enzyme intermediate. These kinetic results are compared and discussed with respect to their implications for the mechanism of GGT-mediated hydrolysis and transpeptidation.

RESULTS AND DISCUSSION

Hammett plots

The rate-limiting step of the reaction catalysed by GGT changes according to the nature of the donor and the acceptor substrates. We synthesized a series of L- and D-γ-glutamylanilides substituted in the *para* position with electron-donating and electron-withdrawing substituents, as shown in Fig. 1. Compounds with L-configuration, containing amide bonds of different strengths, were used as donor substrates for the transpeptidation reaction catalysed by GGT in the presence of saturating concentrations of glycylglycine as acceptor substrate.[16] Apart from *p*-nitroaniline, the anilines released as the first product of the transpeptidation reaction are not strong chromophores. Therefore, for this series of donor substrates the steady-state enzymatic reactions were quenched at different times with trichloroacetic acid and the liberated *p*-anilines were derivatized with sodium nitrite and a naphthyl derivative to give diazo dye complexes detectable at $\sim$560 nm. A Hammett plot with an upward curvature was obtained (circles, Fig. 2), indicating by its non-zero slope that the acylation reaction is the rate-limiting step.[16] The shape of the curve was ascribed to a change in the geometry of the transition state of the concerted rate-limiting step[21,22] in accordance with further evidence from isotope effect, pH–rate profile, Eyring and van't Hoff experiments. The decomposition of the tetrahedral intermediate to liberate the *p*-aniline moiety and to form the putative acyl enzyme intermediate was thus identified as the rate-limiting step of the acylation process.[16]

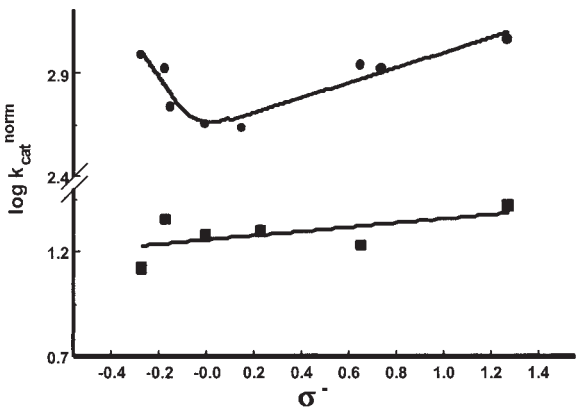

Figure 2. Hammett plots obtained for GGT-mediated transpeptidation from L-γ-glutamylanilides to glycylglycine (circles, Ref. 16) and hydrolysis of D-γ-glutamyl anilides in the absence of acceptor substrate (squares, this work)

The γ-glutamylanilides with D-configuration were used as donor substrates, initially in the absence of any primary amine as an acceptor substrate, in an investigation of the GGT-catalysed hydrolysis reaction. The D-configuration was necessary for this study in order to avoid the autotranspeptidation reaction that is possible with compounds of L-configuration, which can serve as both donor and acceptor substrates.[12] The Hammett plot constructed from the steady-state kinetic data measured for the GGT-mediated hydrolysis of our D-γ-glutamyl anilides (squares, Fig. 2) displays a slope with a ρ value of around zero (0.055 ± 0.067). The Hammett parameter σ^- was used as recommended for anilines as leaving groups;[23] fitting the same kinetic data to other parameters such as σ, σ_R and σ_I or the pK_a of the anilinium ions (i.e. in a Brønsted plot) made no significant difference to the calculated slope (data not shown). From this near-zero slope we may conclude that variation of the strength of the amide bond cleaved during the GGT-catalysed hydrolysis does not lead to significant variation in the steady-state rate of the reaction. Hence it appears that acylation is not the rate-limiting step in the steady-state hydrolysis process. Considering the ping-pong catalytic cycle (Scheme 1) proposed for this enzyme, it seems reasonable to hypothesize that for the hydrolysis reaction, deacylation of the putative acyl enzyme intermediate is rate limiting.

Other results have been reported previously using D-glutamine and D-γ-glutamyl-*p*-nitroanilide as donor substrates under conditions where water served as the acceptor substrate.[24] The reaction with D-glutamine was shown to proceed with rate-limiting acylation, whereas for D-γ-glutamyl-*p*-nitroanilide, deacylation was the rate-limiting step for hydrolysis. Our results are consistent with the latter, and allow us to extend the conclusion that acylation is probably rapid for all γ-glutamylanilides, probably due to leaving group activation. Whether the same is true for the *in vivo* donor substrate glutathione remains to be seen.

Pre-steady-state hydrolysis studies

If the deacylation step of GGT-mediated γ-glutamylanilide hydrolysis is indeed rate limiting in the steady state, as shown above, then one might expect the pre-steady-state reaction to proceed through the initial rapid liberation of the first product, in our case a *para*-substituted aniline, upon predominant conversion of the enzyme to an acylated form, followed by the slow release of the same product during the rate-limiting hydrolysis of the acyl enzyme intermediate during the steady state. A biphasic reaction curve could therefore be expected at short reaction times. No burst of this type has ever been reported for GGT, under conditions where kinetic traces are followed on the second time-scale. It therefore appeared clear to us that pre-steady-state kinetic studies

on the millisecond scale would be necessary to observe the putative burst kinetics.

Pre-steady-state hydrolysis studies were initially carried out using a rapid mix–quench flow apparatus with D-γ-glutamyl-*p*-nitroanilide as substrate. This donor substrate has been well studied in our laboratory[25] and by other groups[24] and releases a chromophore product that is easy to detect. The rapid mix–quench flow apparatus has a dead time of 2 ms and has been used previously with success to follow rapid pre-steady-state enzymatic reactions.[26] Trichloroacetic acid is used as a quench solution, stopping the enzyme reaction and denaturing the enzyme but at the same time lowering the pH and in the current studies protonating the product *p*-nitroaniline to its less visible anilinium form. The liberated aniline was therefore derivatized according to the same protocol used above for Hammett plot. This method poses the advantage of forming a diazo complex with a high extinction coefficient ($57\,\text{mM}^{-1}\,\text{cm}^{-1}$, versus $8.8\,\text{mM}^{-1}\,\text{cm}^{-1}$ for *p*-nitroaniline), resulting in a sensitive detection technique of low concentrations of released product. From this experiment, a distinct biphasic curve was obtained, presenting a burst up to ~50 ms (data not shown). The slope from the rapid phase yields a rate constant of $\sim 80\,\text{s}^{-1}$ for acylation, whereas the slope from the slow phase yields a rate constant of $\sim 4\,\text{s}^{-1}$ for hydrolytic deacylation. These results demonstrated the existence of an initial burst and gave a rough indication of its duration. However, the indirect nature of the detection method, the imprecision of the results and the high concentrations of enzyme (purified in minute quantities from animal tissue[2,16]) necessary for each time point posed serious practical limitations for obtaining duplicate and triplicate measurements leading to precise kinetic data. The experiment was therefore repeated using a stopped-flow spectrometer, a method for continuous kinetic detection that consumes less enzyme.

The *p*-nitroaniline released from D-γ-glutamyl-*p*-nitroanilide during its GGT-mediated hydrolysis was directly observed at 410 nm with relative ease, although its extinction coefficient ($8800\,\text{M}^{-1}\,\text{cm}^{-1}$)[2] is lower than that of its corresponding diazo dye (see above). A typical kinetic trace is shown in Fig. 3. As was observed during the rapid mix–quench experiments, the hydrolysis reaction follows two distinct phases. The initial rapid phase, or burst, lasts for ~65 ms and the slopes for the rapid and the slow phases are 50 ± 5 and $14 \pm 2\,\text{s}^{-1}$, respectively (Table 1). These slopes represent the average of three runs with two different batches of enzyme. When the experiment was repeated using a sample from a different batch of purified GGT, the rate constants obtained were 48 and $16\,\text{s}^{-1}$, respectively, after normalization for specific activity. The similarity of the values obtained for the fast and slow rate constants between batches of enzyme and across different experimental techniques testify to the reproducibility and precision of these results.

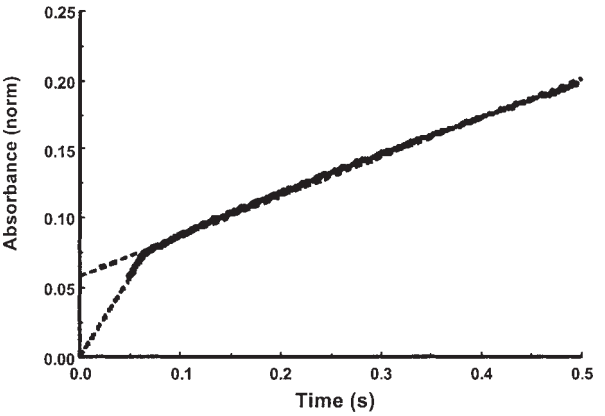

Figure 3. Typical pre-steady-state burst kinetic trace obtained by stopped-flow spectrometry for GGT-mediated hydrolysis of a saturating concentration of D-γ-glutamyl-*p*-nitroanilide (see Table 1 for rate constants)

Table 1. Kinetic constants obtained from pre-steady-state analysis of GGT-mediated hydrolysis and transpeptidation reactions of D-γ-glutamyl-*p*-nitroanilide at pH 8.0, 37 °C

Acceptor substrate	Rapid phase: k_{cat}^{norm} (s^{-1})	Slow phase: k_{cat}^{norm} (s^{-1})	Burst size (μM)
(Water)[a]	50 ± 5	14 ± 2	6.3 ± 1.3
0.3 mM L-methionine[b]	54 ± 10	19 ± 1	6.0 ± 1.2

[a] Hydrolysis in absence of amine acceptor substrate. See Fig. 3; $[\text{GGT}]_0 = 8.0\,\mu\text{M}$.
[b] See Fig. 4; $[\text{GGT}]_0 = 8.0\,\mu\text{M}$.

The burst phase (Fig. 3) represents the rapid pre-steady-state acylation of GGT by the donor substrate D-γ-glutamyl-*p*-nitroanilide. We have used the same donor substrate previously in the presence of a high concentration of glycylglycine, an excellent acceptor substrate, under conditions where acylation is assumed to be rate limiting. In that case, the steady-state rate constant was found to be $297\,\text{s}^{-1}$, almost 6-fold higher than the value determined directly here.[27] It is not clear that this difference is sufficiently significant to warrant speculative explanation based on a difference in mechanisms and/or an additional role of glycylglycine as activator during the acylation step, especially considering the differences in experimental conditions under which the two results were obtained. However, the difference between these values did lead us to investigate directly the burst phase in the presence of an amino acid acceptor substrate, as shown in the next section.

The slow phase of Fig. 3 corresponds to the steady-state hydrolytic turnover of the D-γ-glutamyl acyl enzyme formed in the rapid phase. The rate constant of $14 \pm 2\,\text{s}^{-1}$ determined from the slope of this phase compares well with the k_{cat} value of $14\,\text{s}^{-1}$ found previously for the steady-state hydrolysis of the same compound obtained by visible spectrometry on the second time-scale (data not shown) (R. Castonguay and J. W. Keillor, unpublished observations). It should be noted

that the concentration used in the current stopped-flow experiment (604 μM) is well above the apparent K_M value previously determined for hydrolysis (31 μM), so the favourable comparison is justified and confirms the validity of the current stopped-flow experiments.[28]

When the linear slopes of the slow and fast phases of Fig. 3 are extrapolated to the *y*-axis, the difference in their normalized intercept values of 0.055 ± 0.011 absorbance units is obtained. Using the extinction coefficient of the product *p*-nitroaniline, this absorbance can be converted to a concentration of 6.3 ± 1.3 μM, corresponding to the concentration of the acyl enzyme intermediate formed during the burst phase. This value closely approaches the concentration of GGT used in the experiment, namely 8.0 μM, indicating that essentially all of the free enzyme in solution is converted to acyl enzyme during the rapid pre-steady-state step.

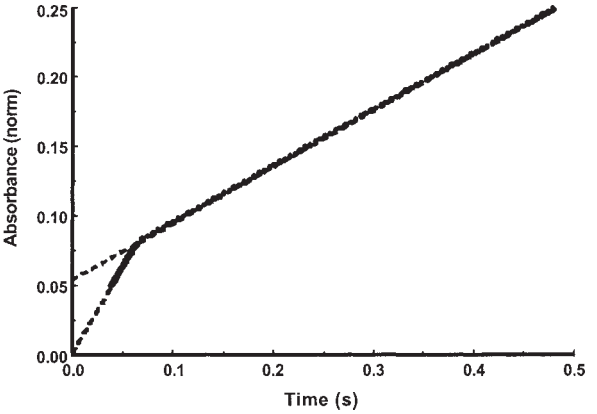

Figure 4. Typical pre-steady-state burst kinetic trace obtained by stopped-flow spectrometry for GGT-mediated transpeptidation from a saturating concentration of D-γ-glutamyl-*p*-nitroanilide to a sub-saturating concentration of L-methionine (see Table 1 for rate constants)

Pre-steady-state aminolysis studies

As noted above, the rate constant determined from the current pre-steady-state kinetic studies for the rapid acylation phase of the reaction of GGT with D-γ-glutamyl-*p*-nitroanilide differs from the value determined from previous steady-state transpeptidation studies under conditions in which acylation was assumed to be rate limiting. Furthermore, one could argue that while the current results indicate that an intermediate is formed during the GGT-mediated hydrolysis reaction, implying a ping-pong mechanism, the transpeptidation reaction could still take place without the formation of such an intermediate, through the direct displacement of *p*-nitroaniline by the α-amino group of an amino acid or dipeptide. For these reasons, it was imperative to perform pre-steady-state kinetic studies similar to those above, but in the presence of an amine acceptor substrate. L-Methionine was chosen as the amino acid acceptor substrate since it is known to be a reasonably efficient acceptor substrate and to display poor affinity for the donor substrate binding site.[12] A concentration of 0.30 mM was used so that the observed steady-state rate of transpeptidation would be noticeably greater than the background hydrolysis reaction, leading to an observable increase in the slope of the slow phase. This concentration is also ~15-fold below the apparent K_M value of L-methionine as an acceptor substrate (4.9 mM)[12] so that the deacylation step of the predominant transpeptidation reaction would not be so fast as to make acylation the rate-limiting step, masking the initial burst.

As can be seen in Fig. 4, the stopped-flow kinetic trace obtained under these conditions is still biphasic. The slope of the initial rapid phase provides a rate constant of $54 \pm 10\ s^{-1}$, corresponding very closely to that obtained in the absence of acceptor substrate (Table 1). This is consistent with the same initial acylation reaction taking place between GGT and D-γ-glutamyl-*p*-nitroanilide, regardless of the presence of amine acceptor substrate. The size of the burst, as extrapolated from the slope of the slow phase, was determined as 0.053 ± 0.011 absorbance units, corresponding to a concentration of 6.0 ± 1.2 μM, approximating the concentration of enzyme used (8.0 μM). This indicates that once again, even in the presence of an amine acceptor substrate, GGT is predominantly acylated by D-γ-glutamyl-*p*-nitroanilide in a rapid initial step, prior to deacylation, confirming the purported ping-pong mechanism.[12] The slope of the slow phase, corresponding to the steady state turnover of the acyl enzyme in the presence of L-methionine, gives a rate constant of $19 \pm 1\ s^{-1}$. This is significantly higher than was observed in the absence of amine, as is expected for the more rapid transpeptidation reaction that predominates over hydrolysis.[27]

Ping-pong mechanism

As discussed previously, a modified ping-pong mechanism has been proposed for the catalysis of GGT.[12] One line of evidence for this mechanism is the construction of Lineweaver–Burk plots displaying parallel curves when the concentrations of both donor and acceptor substrates are varied. Although this has been previously obtained for GGT,[29] some authors still contend that the mechanism is sequential.[17] Other evidence in favour of a ping-pong mechanism would be the observation of the formation of an acyl enzyme intermediate. Elce reported the formation of an acylated enzyme, but its structure was different than the γ-glutamyl enzyme that would be formed under physiological conditions.[19] In the current study, the donor substrate used acts like the *in vivo* substrate glutathione in that the same γ-glutamyl moiety is transferred to the active-site nucleophile without any previous modification of the enzyme. We have observed the formation of a putative acyl enzyme intermediate for the GGT-mediated hydrolysis reaction of D-γ-glutamyl-*p*-nitroanilide, as

demonstrated by the biphasic curve shown in Fig. 3. An intermediate was also formed, with the same rate constant, in the burst phase of the transpeptidation reaction with L-methionine, as shown in Fig. 4, under conditions where deacylation was still rate limiting in the steady state. In both cases, the acylating agent used is admittedly an analogue of glutathione, the native substrate. D-γ-Glutamyl-*p*-nitroanilide is commonly used as a substrate analogue because the release of the initial product, *p*-nitroaniline, is easily detected spectrometrically. Although it is unlikely that the use of such an activated donor substrate would give rise to the formation of an acyl enzyme intermediate different to that formed in the native reaction, since the kinetic behaviour of γ-glutamylanilides and glutathione have been demonstrated previously,[2] it may be prudent to verify the formation of an acyl enzyme intermediate with the native donor substrate, glutathione. The use of a rapid mix–quench apparatus offers the advantage of flexibility of analysis and is especially useful for kinetic reactions of substrates that do not possess a chromophore and cannot be followed directly, by stopped-flow spectrometry for example. We have recently developed an HPLC-based assay for the discontinuous kinetic study of GGT substrates and products such as glutathione and γ-glutamyl di- and tripeptides.[30] The application of this analytical method to the confirmation of the ping-pong mechanism with glutathione is in progress.

Acyl enzyme product studies

As mentioned above, other research groups have identified and isolated an acyl enzyme intermediate by preincubating the free enzyme with the chemical modifier *N*-acetylimidazole.[19,20] Both groups reported the partially inhibited hydrolysis of either [^{14}C]glutathione or L-γ-glutamyl-*p*-nitroanilide and the accumulation of a supposedly covalent intermediate. However, the nature of the bond formed between the purported γ-glutamyl moiety and the nucleophile differs between the reports. Experiments carried out by Elce's group showed that the bond was stable to urea, hydroxylamine, performic acid and acidic conditions. In basic conditions, the intermediate was found to hydrolyse slowly, leading the authors to propose that an amide bond was formed in the active-site of GGT. Meister's group performed the same experiments and showed that the acyl enzyme could undergo hydrolysis in the presence of hydroxylamine or guanidinium ions and proposed that the bond formed with the active-site nucleophile was an ester bond. However, in both of the above studies, the acylated amino acid residue was not identified. More recent studies have shown that the active-site nucleophile of *E. coli* GGT is the N-terminal threonine residue of the small subunit,[3] apparently confirming that an ester bond would be present in the reaction with γ-glutamyl derivatives. Since this threonine residue is conserved among all species, the acyl enzyme whose formation with rat kidney GGT was observed in the current study may also be a γ-glutamyl ester. Characterization of samples of γ-glutamyl enzyme from our rapid mix–quench studies, by enzymatic digestion and mass spectral analysis, is in progress.

In conclusion, we have tested D-γ-glutamylanilides substituted in the *para*-position as donor substrates for the hydrolysis reaction of rat kidney γ-glutamyl transpeptidase. A Hammett plot with a ρ value of around zero was obtained, indicating that hydrolytic deacylation, and not acylation, is the rate-limiting step in the steady-state reaction. Pre-steady-state kinetic studies were then carried out with D-γ-glutamyl-*p*-nitroanilide, using both a rapid mix–quench apparatus and a stopped-flow spectrometer. A biphasic curve was obtained by both methods, typical of the rapid formation of an intermediate prior to its rate-limiting turnover. This intermediate is proposed to be an acyl enzyme intermediate and further experiments in the presence of L-methionine, an amine acceptor substrate, also confirmed its formation during the catalytic transpeptidation reaction. The direct observation of this intermediate in GGT-mediated hydrolysis and transpeptidation reactions is consistent with the proposed ping-pong mechanism of this enzyme of broad importance in human physiology.[12]

EXPERIMENTAL

Materials

The donor substrates L- and D-γ-glutamylanilides substituted in the *para*-position were synthesized as described previously.[16] GGT was purified from rat kidney, as previously reported,[16] and kept in solution for the Hammett studies or lyophilized for the rapid mix–quench and stopped-flow experiments. For the latter treatment, the enzyme was resuspended in 0.1 M Tris–HCl, pH 8.0, buffer. Tris buffer was obtained from Bio-Rad and trichloroacetic acid, sodium nitrite, ammonium sulfamate and *N*-(1-naphthyl)ethylenediamine·2HCl were purchased from Aldrich. An Ultrospec 2000 UV–visible spectrophotometer (Pharmacia Biotech) was used for the Hammett experiments. The absorbance values for the rapid mix–quench samples were read on a Cary 100 Bio instrument (Varian). The rapid mix–quench flow apparatus was a KinTek Chemical-Quench-Flow Model RQF-3 (KinTek). The stopped-flow spectrometer was an SX.18MV Stopped-Flow Reaction Analyser (Applied Photophysics) having an optical pathlength of 10 mm and under the control of a Risc processor running the software Pro-K.

Methods

Hammett plot. The experimental procedures used for kinetic analysis of the steady-state GGT-mediated

hydrolysis and transpeptidation reactions were published previously for L-γ-glutamylanilide compounds.[16] The only difference from the protocol used in the current study is the use of donor substrates with D-configuration, at concentrations in the range 1–4000 μM.

Rapid mix–quench. The rapid mix–quench apparatus was calibrated previously with radioactive solutions as described by the manufacturer and equilibrated to 37 °C using a circulating water-bath (Neslab). A 2 mM solution of the donor substrate D-γ-glutamyl-*p*-nitroanilide was prepared in 0.1 M Tris–HCl, pH 8.0, in order to give a final concentration of ~1 mM in the reaction loop. The enzyme was resuspended to a concentration of 0.86 mg ml^{-1} in the same buffer. Upon initiation of a reaction by the rapid mix–quench apparatus, three syringes were pushed. Two contained 0.1 M Tris–HCl, pH 8.0, buffer to push the enzyme and the substrate into the mixing loop. The third contained the quench solution, which was 40% trichloroacetic acid in the current studies. The enzyme and the donor substrates were placed in 1 ml syringes and used to fill the reaction loops, having volumes of 13.8 and 14.6 μl, respectively. Kinetic data were collected at various times according to the procedure described in the manufacturer's manual. Data points were thus obtained in duplicate at 2, 10, 20, 50, 500 and 2000 ms. Blank experiments were performed at 2 and 2000 ms by replacing the enzyme solution in the reaction tube with buffer. The appropriate amount of enzyme was then added to the quenched reaction mixture and analysed as described below.

For each data point, the volume of recovered quenched solution was known precisely. Buffer was then added such that the latter represented one quarter of the final volume. An identical volume of 4 mg ml^{-1} sodium nitrite solution was added and the solution was allowed to stand for 3 min. An identical volume of 20 mg ml^{-1} ammonium sulfamate was then added, followed by incubation for 2 min. Finally, a 2-fold larger aliquot of 1.5 mg ml^{-1} *N*-(1-naphthyl)ethylenediamine solution was added and the solution was allowed to stand for 10 min prior to measuring its absorbance on the Cary spectrometer at 560 nm. The values obtained were divided by the appropriate extinction coefficient (0.057 μM^{-1} cm^{-1}), and multiplied by the necessary dilution factor to obtain the concentration of the aniline present in the initial reaction volume of 28.4 μl (prior to dilution by quench solution and diazotization). The biphasic kinetic curve obtained was analysed by linear regression using the software Axum 5.0. The slopes of each phase were transformed to k_{cat}^{norm} values by dividing by the final concentration of the enzyme in the reaction mixture (6.2 μM) and by normalizing the value to a specific activity of 837 U mg^{-1}.

Stopped-flow. The donor substrate D-γ-glutamyl-*p*-nitroanilide was dissolved to give a concentration of 6.64 mM in 0.1 M Tris–HCl, pH 8.0, buffer. Enzyme was resuspended to a concentration of ~0.6 mg ml^{-1}. A 1.0 ml syringe containing the enzyme and a 0.1 ml syringe containing the donor substrate were fitted into the stopped-flow apparatus, thermostatted at 37 °C, giving a mixing ratio of 10:1 in the stopped-flow cell, and a final GGT concentration of around 8.0 μM. Blanks were performed by alternately replacing the enzyme and the donor substrate by buffer. A series of six acquisitions were carried out for each test. Typically, the first three acquisitions were necessary to fill the lines of the apparatus with the proper solutions, whereas the last three were reproducibly equivalent and averaged together as an experimental result. Given the 10-fold difference in the size of syringes used for initiating the stopped-flow reaction, mixing times were visibly increased beyond the dead time of the instrument and kinetic data were collected after equilibration. The absorbance over the 10 mm pathlength was recorded at 410 nm for 1000 data points on a logarithmic time scale over 10 s for each run. The absorbance values were then analysed using the software Axum 5.0 to determine the values of the linear slopes for each phase of the trace, using the extinction coefficient of 8.8 mM^{-1} cm^{-1}. These slopes were transformed into k_{cat}^{norm} values as described above for the rapid mix–quench experiments.

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