Kinetic Studies of Rat Kidney γ-Glutamyltranspeptidase Deacylation Reveal a General Base-Catalyzed Mechanism†

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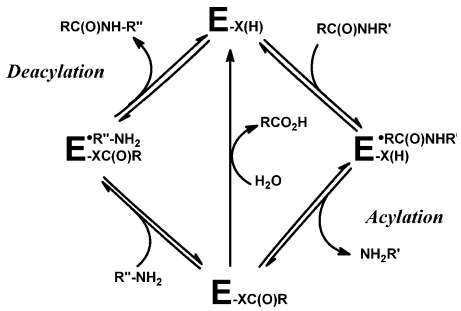
ABSTRACT: The enzyme γ-glutamyltranspeptidase (GGT) is critical to cellular detoxification and leukotriene biosynthesis processes, as well as amino acid transport in kidneys. GGT has also been implicated in many important physiological disorders, including Parkinson's disease and inhibition of apoptosis. It binds glutathione as a donor substrate and initially forms a γ-glutamyl−enzyme complex that can then react with a water molecule or an acceptor substrate (usually an amino acid or a dipeptide) to form glutamate or a product containing a new γ-glutamyl−isopeptide bond, respectively, thus regenerating the free enzyme. Despite its important role in human physiology, the mechanisms of the reactions catalyzed by GGT are not well-known, particularly with respect to the deacylation step. We have synthesized a series of methionine amide derivatives whose α-ammonium groups have different pK_a values. By using these compounds as acceptor substrates for GGT, we have constructed a Brønsted plot and obtained a good correlation for $\log(k_{cat,b}^{norm}/K_b)$ *versus* $pK_a^{NH^+}$ with a slope β_{nuc} of 0.84, consistent with a rate-limiting nucleophilic attack of the substrate amine on the acyl−enzyme intermediate. Isotope effect studies have shown that there is a proton in flight at the transition state, consistent with concerted deprotonation of the nucleophilic amine effected by an unidentified general base. A bell-shaped pH−rate profile has also been obtained for the deacylation step, reflecting the pK_a values of the acceptor substrate (and/or that of a general base residue) and of a putative general acid that may be necessary for reprotonation of the active site nucleophile upon regeneration of the free enzyme. These data allow us to propose for the first time a detailed mechanism for this important step of the GGT-mediated reaction and to speculate about the origin of its acceptor substrate specificity.

The enzyme γ-glutamyltranspeptidase (GGT,[1] EC 2.3.2.2) is a highly glycosylated heterodimeric enzyme found in mammals and plants (*1*, *2*). In mammals, it is found in brain, liver, pancreas, and especially in kidneys (*3*−*5*). It plays a role in cellular detoxification through formation of mercapturic acids and confers resistance against antitumor drugs (*6*). It is also implicated in the biosynthesis of leukotriene D that mediates bronchoconstriction in asthma (*7*, *8*) and in amino acid transport in kidneys (*9*). It also serves as a hepatobiliary marker in routine medical tests (*4*, *10*). GGT has also been implicated in many physiological disorders, such as Parkinson's disease (*5*), diabetes (*11*), and inhibition of apoptosis (*12*, *13*). It has been proposed that GGT is a key element in all processes against oxidative stress due to the physiological role of its *in vivo* substrate, glutathione (*14*).

GGT uses glutathione as an acyl donor substrate and transfers its γ-glutamyl moiety to acceptor substrates, such as amino acids or dipeptides, to form a product containing a new isopeptide bond. This transamidation role is very important for amino acid transport in the kidney (*9*, *15*). The reaction catalyzed by GGT is known to proceed through a modified ping-pong mechanism, as shown in Scheme 1 (*1*, *16*). The enzyme binds its donor substrate, is transiently acylated by its γ-glutamyl moiety, and releases the first reaction product (cysteinylglycine, in the case of glutathione) during the acylation step. The resulting γ-glutamyl acyl−enzyme intermediate can then react with either water (hydrolysis) or an acceptor substrate (typically an amino acid or a dipeptide) in a deacylation step to form either glutamate or a transpeptidated product, respectively. The transpeptidation reaction is presumed by many to be the most important reaction *in vivo* due to the presence of high concentrations

Scheme 1



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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; GGT, γ-glutamyltranspeptidase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ntn-hydrolase, N-terminal nucleophile hydrolase; SKIE, solvent kinetic isotope effect; TGase, transglutaminase; Tris, tris(hydroxymethyl)aminomethane.

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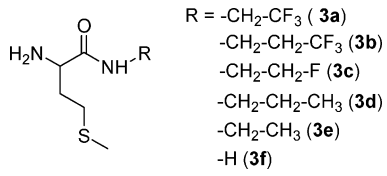


FIGURE 1: Structure of the L-methionine derivatives used as acceptor substrates for the deacylation step of GGT (see the Supporting Information for synthesis details).

of amino acids in kidney (*17*), although others are proponents of the physiological importance of the hydrolysis reaction (*18*).

We have previously shown (*19*) that the mechanism of the acylation step (at least from γ-glutamylanilides) implicates the formation of a tetrahedral intermediate, upon the nucleophilic attack by an unidentified amino acid on the γ-carbonyl group of the substrate. This is then followed by the concerted general acid protonation of the leaving group and the cleavage of the C−N bond in the decomposition of the tetrahedral intermediate. However, the mechanism of the deacylation step, a distinguishing feature of the mechanism of transpeptidase enzymes, has not been studied as extensively and merits a more detailed investigation.

Certain amino acid dipeptides have been shown (*20*) to serve as acceptor substrates. Taking this substrate affinity into account, we have synthesized amide derivatives of methionine bearing different parent amine groups in an effort to prepare a series of acceptor substrates with similar structures with varied pK_a values of their Met α-ammonium groups (Figure 1). The kinetic data measured for the deacylation reactions of these acceptor substrates, including their Brønsted correlation, pH−rate studies, and kinetic isotope effect experiments are presented herein. In the discussion of these results, a mechanism is proposed that sheds more light on the reactivity and specificity of GGT.

EXPERIMENTAL PROCEDURES

Materials

Rat kidney GGT was isolated and purified as previously published (*19*). L- and D-γ-glutamyl-*p*-nitroanilide (donor substrates for GGT) were synthesized according to a published protocol (*21*). Glycylglycine, MES, MOPS, and CHES buffers and L-methioninamide (compound **3f**) were purchased from Aldrich. All kinetic data were obtained using a Cary 100 Bio UV−vis spectrophotometer (Varian). Potentiometric titrations were performed using a gel-filled Accumet combination electrode with an Ag/AgCl reference (Fisher).

Methods

Synthesis of Methionine Derivatives. The synthesis of compounds **3a**−**3e** is described in detail in the Supporting Information.

Titration Experiments. The pK_a values of compounds **3a**−**3f** were determined by titration with a Mettler Toledo DL53 autotitrator apparatus. A 0.2 M solution of each compound was made up in 55 mM KCl, corresponding to the approximate ionic strength of the enzymatic reaction mixture. The titration of this solution was carried out using 0.1 N NaOH. Titration curves were performed in duplicate.

Steady-State Kinetics. A 0.1 M solution of each compound (**3a**−**3f**) was prepared in 0.1 M Tris-HCl buffer (pH 8.0). Cuvettes were prepared containing different concentrations (5−150 μM) of the donor substrate D-γ-glutamyl-*p*-nitroanilide (from a 5 mM stock solution), and the volumes were increased to 1 mL with 0.1 M Tris-HCl buffer (pH 8.0). Kinetic reactions were initiated by the addition of ∼39 milliunits of rat kidney GGT. The release of *p*-nitroaniline was followed spectrophotometrically at 410 nm (ϵ = 8800 M^{-1} cm^{-1}). To study the effect of the acceptor substrate on the steady-state rate of release of *p*-nitroaniline, the same cuvettes were prepared with a fixed concentration of the acceptor substrate (compounds **3a**−**3f**) from 4 to 20 mM. To correct for nonenzymatic hydrolysis of the donor substrate, kinetic runs were performed as described above but without enzyme. Initial rates of the transpeptidation reaction were determined by dividing the linear slopes of absorbance *versus* time, measured over the first <10% of the reaction, by the appropriate extinction coefficient. The initial rates thus determined at each concentration of donor and acceptor substrates were then used to draw linear Lineweaver−Burk plots. Concentrations were chosen to distribute data points relatively evenly over the reciprocal *x*-axis; weighted or unweighted analysis gave approximately the same slope values. Equation 1 was used to obtain the appropriate kinetic constants:

$$\frac{1}{v} = \frac{K_h(1 + [B]/K_i)}{V_h[A]} + \frac{1 + [B]/K_b}{V_h(1 + [B]/K_{iab})} \qquad (1)$$

where v is the rate of the reaction, [A] is the concentration of the donor substrate, [B] is the concentration of the acceptor substrate, K_b is the Michaelis constant for the molecule acting as an acceptor, K_h is the Michaelis constant for the hydrolysis reaction, V_h is the maximal velocity for the hydrolysis reaction, V_b is the maximal velocity for the transfer reaction, K_i is proposed to be the inhibition constant for the acceptor bound at the donor site, and K_{iab} is the partition coefficient, equal to $(K_bV_h)/V_b$ (*16*).

The *y*-axis intercept for the Lineweaver−Burk plot in the absence of an acceptor substrate (i.e., the hydrolysis reaction) was set to be int_0, and all the other intercepts were taken to be int_x. The subsequent replot of $(int_0 - int_x)^{-1}$ as a function of the reciprocal of the acceptor substrate concentration then gave $-1/K_{iab}$ as the abscissa intercept (*16*). Then, a value of V_b/K_b was found for each acceptor substrate that was used and transformed into $k_{cat,b}^{norm}/K_b$ by dividing by the concentration of enzyme in each cuvette and by normalizing the specific activity of the enzyme used in each experiment to 837 units/mg (*19*). The graph of $\log(k_{cat,b}^{norm}/K_b)$ as a function of the pK_a of each acceptor substrate, the Brønsted plot, was fitted by linear regression, as were all the other plots, using Axum 5.0 software. The experimental error in the logarithmic data was determined by propagating and calculating the effect of the error measured for the partition coefficient (K_{iab}). All kinetic experiments were carried out in duplicate.

pH−Rate Profile Studies. Experiments were performed as described in Steady-State Kinetics with L-methionylpropylamide (**3d**) in buffered solutions at different pHs. All buffer solutions were prepared at a concentration of 0.1 M (MES for pH 6.25, MOPS for pH 7.0, 7.25, and 7.50, Tris for pH

Table 1: Kinetic Parameters ($k_{cat,b}^{norm}/K_b$) Obtained According to eq 1 (see Experimental Procedures) for GGT-Mediated Transpeptidation from D-γ-Glutamyl-*p*-nitroanilide to Different L-Methionine Derivatives (pH 8.0 and 37 °C) and the Ammonium pK_a Values of Their Respective Conjugate Acids

acceptor substrate	structure of the variable moiety	$\log(k_{cat,b}^{norm}/K_b)^a$	ammonium pK_a[b]
L-methionyl(2,2,2-trifluoroethylamide) (**3a**)	CH_2CF_3	5.24 ± 0.03	7.09
L-methionyl(3,3,3-trifluoropropylamide) (**3b**)	$CH_2CH_2CF_3$	5.378 ± 0.004	7.30
L-methionyl(2-fluoroethylamide) (**3c**)	CH_2CH_2F	5.51 ± 0.02	7.37
L-methionylpropylamide (**3d**)	$CH_2CH_2CH_3$	5.52 ± 0.02	7.40
L-methionylethylamide (**3e**)	CH_2CH_3	5.48 ± 0.01	7.41
L-methioninamide (**3f**)	H	5.85 ± 0.02	7.50

[a] Results are from duplicate experiments normalized to a specific activity of 837 units/mg. [b] Measured titrimetrically in 55 mM KCl at 25 °C (see Experimental Procedures). The experimental error is estimated to be 0.01 unit.

8.0, 8.5, and 8.75, and CHES for pH 9.0 and 9.5). The extinction coefficient of *p*-nitroaniline used at pH 6.25 was measured by preparing a linear standard curve of the absorbance at 410 nm as a function of the concentration of *p*-nitroaniline (0−100 μM) in 0.1 M MES buffer at pH 6.25 and determined to be 7800 M^{-1} cm^{-1}. All other extinction coefficients that were used were taken from previously published material (*19*). Kinetic experiments were generally performed in duplicate, except for those of the observed plateau at pH 7.0, 8.75, and 9.0. The stability of GGT at these pHs has been previously verified (*19*).

Isotope Effect Studies. Kinetic experiments were carried out as described in Steady-State Kinetics with compounds **3c**, **3d**, and **3f**. All solutions were prepared in D_2O, and the pD was adjusted to 8.0 (i.e., a reading of pH 7.6 using a glass electrode) (*22*). The extinction coefficient of *p*-nitroaniline in D_2O was determined as described in pH−Rate Profile and confirmed to have the same value as that in H_2O.

RESULTS AND DISCUSSION

Design of Acceptor Substrates. A study of the effect of the pK_a of the acceptor substrate on the rate of the deacylation step would provide useful information about the mechanism of this step and the role of a putative general acid/base residue. For these studies, rat kidney was judged to be an appropriate source of GGT, because of its availability and high degree of homology with the human enzyme (*1*). The specificity of rat kidney GGT for its acceptor substrates allows the use of very slightly modified amino acids. The binding site for the acceptor substrate of GGT has been proposed to be composed of two subsites, each able to recognize amino acids of the L-configuration only (*16*). It has been suggested that these subsites are occupied by the cysteinylglycine moiety of the donor substrate glutathione during the acylation step (*20*, *23*). Primary amines (other than amino acids), including anilines, are not well recognized and do not act efficiently as acceptor substrates (*16*, *20*). The amino acids that serve most efficiently as acceptor substrates are L-cystine, L-glutamine, L-methionine, and L-alanine (*16*). Sterically hindered L-amino acids or D-amino acids do not function as acceptor substrates. Dipeptides are better substrates than simple amino acids; it has been suggested that this may be due to the decreased pK_a of their ammonium groups and their occupancy of the second subsite (*24*). With respect to this second subsite, glycine has been shown to be the best amino acid for conferring affinity to dipeptide substrates (*20*).

We chose L-methionine as the parent compound of our series of acceptor substrates since it is known to be a good acceptor substrate for GGT (K_M = 4.7 mM), although less efficient than dipeptides (*16*). The efficient recognition of L-methionine by GGT can be explained intuitively since it somewhat resembles the Cys residue of glutathione (or of a corresponding glutathione S conjugate) of the native donor substrate that may be bound in this subsite (*25*). Furthermore, L-methionine has been shown to be a poor inhibitor in the donor substrate binding site (K_i = 26.9 mM), compared with other simple amino acids (*16*). These characteristics ensure that the kinetic results obtained with derivatives of this amino acid are pertinent to the enzymatic deacylation reaction and not to an adventitious side reaction. Moreover, the higher affinity of L-methionine and its derivatives for the targeted acceptor substrate binding site, as opposed to the donor substrate binding site, makes the kinetic analysis less ambiguous. For these reasons, amide derivatives of L-methionine bearing different parent amine groups were synthesized, giving a series of acceptor substrates with similar structures wherein the pK_a values of their Met α-ammonium groups were varied as much as possible (Figure 1).

*Synthesis of Compounds **3a**−**3e**.* The straightforward synthesis of compounds **3a**−**3e** was successfully carried out as shown in detail in the Supporting Information. The syntheses diverged from a common synthetic intermediate, *N*-Boc-L-Met, simplifying the preparation of the series of acceptor substrates. Coupling with primary amines of varying basicity, followed by deprotection of the α-amino group, provided compounds **3a**−**3e** whose purity was verified prior to their use in subsequent kinetic studies. 3,3,3-Trifluoropropylamine was prepared from the corresponding bromide as shown in the Supporting Information. Compound **3f**, L-methioninamide, was obtained from commercial sources.

Determination of α-Ammonium pK_a Values. The pK_a values of compounds **3a**−**3f** were found to span a narrow range between 7.09 and 7.50, as shown in Table 1. Obviously, the inductive effects of the electron-withdrawing substituents of the parent amines on the pK_a of the α-amino groups are attenuated by the distance between the amide and the ammonium group. The precision of the potentiometric determination of the pK_a values was therefore very important. For all measured values, the relative error was determined to be ∼0.1%, indicative of the reproducibility of the experimental values. The pK_a values used in subsequent correlation studies (*vide infra*) were measured directly in this study, under conditions similar to those used for kinetic studies, rather than taken indirectly from elsewhere in the literature, since the conditions and precision of other reported pK_a values were found to vary widely.

Kinetic Studies of the Deacylation Step. To ensure that our kinetic results are pertinent specifically to the deacylation step, certain control experiments were performed. The best acceptor substrate for GGT is glycylglycine (*3*). However, to study the deacylation step, the acylation step of the catalytic cycle must be faster and not rate-limiting. Some preliminary pre-steady-state studies of the hydrolysis reaction mediated by rat kidney GGT have been carried out using D-γ-glutamyl-*p*-nitroanilide as the donor substrate (*26*). These studies demonstrate an initial burst of *p*-nitroaniline product up to 50 ms. Also, it is widely known that the hydrolysis is rate-limiting relative to the acylation step with γ-glutamyl-*p*-nitroanilide as the donor substrate (*23*). For the purposes of this study, it is only important that these results confirm that deacylation is the rate-limiting step for the hydrolysis reaction, and that under the conditions of these kinetic studies, the linear kinetics observed correspond to the slow deacylation phase (*27*). It is therefore reasonable to approximate that the acyl−enzyme intermediate is the major form of the enzyme in the reaction mixture in this case. Furthermore, the use of an amino acid such as L-methionine, which is a reasonably efficient acceptor substrate, but far from the most rapid, will ensure that even though the ensuing transpeptidation reaction is accelerated over the slower hydrolysis reaction, deacylation will nevertheless remain the rate-limiting step. Finally, the concentration of the acceptor substrate used in all experiments was low (below their K_M value), ensuring that the deacylation step was in all cases rate-limiting, as clearly shown in the results below. The donor substrate used was d-γ-glutamyl-*p*-nitroanilide, and not its L-isomer, to avoid the autotranspeptidation reaction that is possible with the latter (*3*) and to ensure once again that the kinetically relevant observed reaction was indeed the deacylation step of transpeptidation of our synthetic methionine derivatives.

To obtain kinetic data that are relevant to only the aminolysis step of the transamidation reaction, and not to the combined aminolysis and hydrolysis reactions of the acyl−enzyme intermediate, mathematical manipulations of the data that are somewhat more complex than typical steady-state analyses were necessary. Reaction mixtures were prepared as described in Experimental Procedures. For each substrate, a set of cuvettes were prepared at various donor substrate concentrations and fixed acceptor substrate concentrations. A typical example of the Lineweaver−Burk plot obtained for each substrate is shown in Figure 2. As explained in Experimental Procedures, the reciprocal of the difference between the *y*-axis intercept obtained for the hydrolysis reaction (0 mM acceptor substrate, int_0) and the intercept obtained for a transpeptidation reaction at a fixed concentration of acceptor (int_x; see Experimental Procedures) was replotted as a function of the reciprocal of the concentration of acceptor substrate B ($1/int_0 - int_x$ *vs* 1/[B]; see Figure 3). This gives an abscissa intercept value corresponding to the reciprocal of the partition coefficient, K_{iab}. The $k_{cat,b}^{norm}/K_b$ kinetic parameters also obtained in this manner for each substrate are given in Table 1.

From the results presented in Table 1, it is possible to determine the K_b value (the true K_M for the acceptor substrate) from the intercept value of eq 1. The K_b values for these substrates were found to be between 30 and 39 mM, except for that of compound **3f**, L-methioninamide (K_b = 16 mM). These values are significantly higher than the concentrations used for each compound acting as an acceptor substrate (which were less than 20 mM for all except L-methioninamide, which was less than 8 mM), ensuring that deacylation was always the rate-limiting step. Because of the successive mathematical manipulations of the raw rate data, the kinetic parameters thus determined are known to be susceptible to significant experimental error (*16*); however, given the reproducibility of multiple measurements, their values are nevertheless informative.

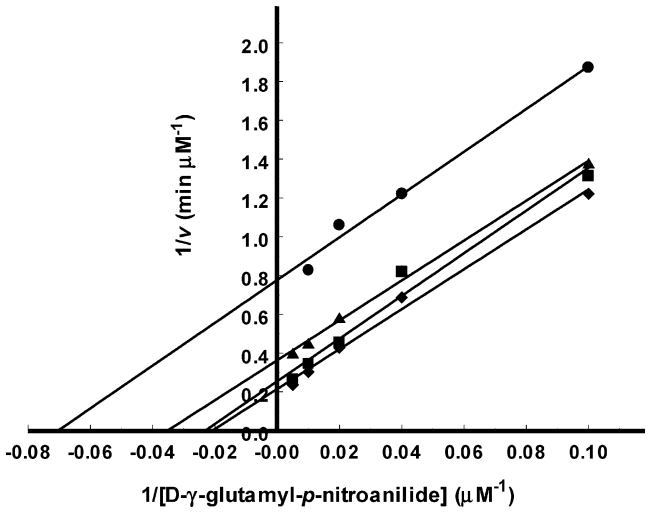


FIGURE 2: Lineweaver−Burk plot of the rate of the transpeptidation of D-γ-glutamyl-*p*-nitroanilide as a donor substrate by GGT in the absence and presence of different constant concentrations of L-methionyl(2-fluoroethylamide) (**3c**) as an acceptor substrate: (●) 0 mM acceptor substrate, (▲) 5 mM, (■) 10 mM, and (◆) 15 mM. For experimental details, see *Materials and Methods*.

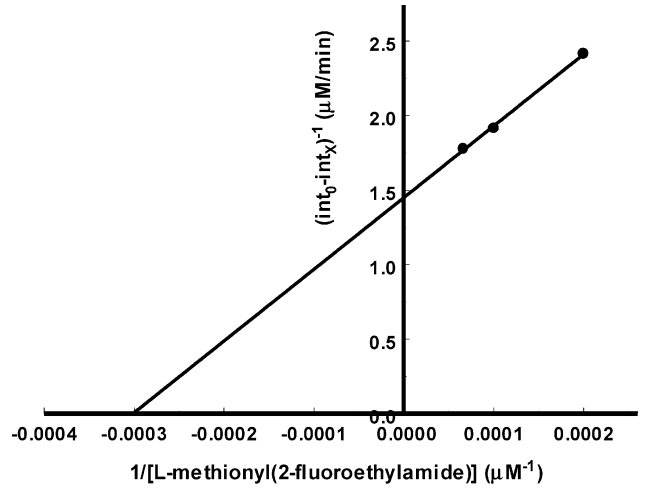


FIGURE 3: Replot curve of the reciprocal of the difference of the intercept of the hydrolysis reaction in Figure 2 (int_0) and the intercept at different concentrations of the acceptor substrate (int_x) as a function of the reciprocal of the concentration of this substrate. For the calculations, see Experimental Procedures.

It is also noteworthy that the slopes of Figure 2 are the same, consistent with the negligible inhibition (that is, high K_i value in eq 1) of the transpeptidation reaction due to the possible binding of L-methionine derivatives in the donor substrate binding site, as discussed previously. Moreover, we have recently shown that a GGT inhibitor, whose lateral chain contains a heteroatom at the γ-position and an esterified

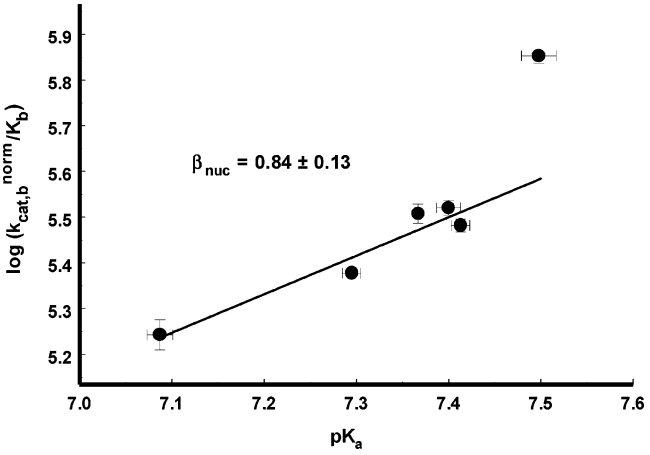


FIGURE 4: Brønsted plot of the kinetic data for the reaction of 1 mM D-γ-glutamyl-*p*-nitroanilide in the presence of a series of L-methionine derivatives acting as acceptor substrates for rat kidney GGT at 37 °C and pH 8.0. Without the data at the pK_a of 7.50, the slope β_{nuc} is equal to 0.84 ± 0.13. All results were obtained in duplicate.

α-carboxylate group, is more likely to be bound at the acceptor substrate binding site than the donor substrate binding site (*28*). Glycinamide and L-methionine ethyl ester have previously been shown to act as good acceptors (*20*, 25). Taken together, it is reasonable to conclude that our synthetic L-methionine amide derivatives are predominantly bound in the acceptor binding site and that inhibition at the donor binding site is negligible, if not nonexistent.

Great care was taken to ensure that the kinetic constants obtained in this study are *true*, and not apparent, values. While a sizable amount of steady-state kinetic data for the deacylation step has been published for GGT, much has been without rigorous consideration of the enzymatic "background" reaction of deacylation due to hydrolysis (*16*). Although this hydrolysis reaction is negligible in the presence of many acceptor substrates (glycylglycine, for example), it becomes relatively increasingly important for less efficient amino acid acceptor substrates. It was therefore essential in this study to take the hydrolysis reaction into account, as described above, so that the measured kinetic parameters can be analyzed in terms of the mechanism of aminolytic deacylation. Finally, since values for $k_{cat,b}^{norm}/K_b$, deriving directly from this kinetic analysis, are known to be more precise values, because there are fewer mathematical manipulations involved than in calculating k_{cat} or K_M (*16*), the former ratio values were used for subsequent relative rate analyses.

Brønsted Correlation for the Deacylation Step. The kinetic data from Table 1 were used to study the correlation between the efficiency of the enzymatic deacylation step as a function of the pK_a of the conjugate acid form of the different L-methionine derivatives used as acceptor substrates. The results are shown in Figure 4.

The pK_a values for this series of L-methionine derivatives span a narrow range over which a clear correlation with the rate data can be observed, because of the precision and reproducibility of the kinetic measurements. (Although it would have been preferable to have a wider range of pK_a values to study, we were unable to further perturb the pK_a values of the methionine derivatives without introducing significant secondary steric effects.) The observed correlation is not due to a simple steric effect of the amide on each acceptor compound, as is obvious from the relatively minor difference in $\log(k_{cat,b}^{norm}/K_b)$ between the homologues L-methionylpropylamide (**3d**) and L-methionylethylamide (**3e**) or L-methionyl(2,2,2-trifluoroethylamide) (**3a**) and L-methionyl-(3,3,3-trifluoropropylamide) (**3b**). Rather, the correlation is clearly due to a pK_a dependence of the rate data. The value of the slope measured for this correlation [$\beta_{nuc} = 0.84 \pm 0.13$ ($r = 0.932$)] does not take into account the data point at a pK_a of 7.50 for L-methioninamide (**3f**). As stated above, this compound was found to have a K_b value well outside the range of the other acceptor substrates, as reflected in its $\log(k_{cat,b}^{norm}/K_b)$ value and probably because its structure is significantly different from those of other members (**3a**−**3e**) of the series. It is conceivable that **3f**, being a primary amide, is bound in a more productive fashion or with greater affinity than the secondary amides **3a**−**3e**. Thus, L-methioninamide is not considered to be in the same series as the other compounds, but reinforces nevertheless the general positive slope observed for the correlation with all of the L-methionine derivatives. Finally, since the kinetic data were obtained at pH 8.0, above the pK_a of all the compounds tested, no significant correction for the proportion of the amine in a specific protonation state was required (*24*, *29*).

As mentioned earlier, the measured $k_{cat,b}^{norm}/K_b$ values are taken to be more precise than the corresponding $k_{cat,b}^{norm}$ parameters, because of the additional calculations that are necessary to isolate the K_b values (*16*). We were nevertheless curious to see if a Brønsted correlation also exists for $\log(k_{cat,b}^{norm})$ *versus* the pK_a of the conjugate acid of the acceptor substrate amine. A β_{nuc} value of 0.61 ± 0.12 ($r = 0.900$) was obtained for this correlation (results not shown), confirming the general positive slope for this correlation, but suggesting that a tighter correlation exists with $\log(k_{cat,b}^{norm}/K_b)$, as shown in Figure 4. This correlation is consistent with the nucleophilic attack of the amine acceptor substrate on the proposed acyl−enzyme ester intermediate to form a tetrahedral intermediate during the rate-limiting event of the deacylation step, and probably not the decomposition of this intermediate, which would presumably exhibit a better correlation with $k_{cat,b}^{norm}$ alone (*30*).

In general, a positive Brønsted β_{nuc} slope for the aminolysis of an intermediate means that the nucleophilic attack on the intermediate leads to the development of positive charge on the amine nitrogen at the transition state and that its nucleophilicity is important in the deacylation process. In contrast, a negative slope is explained by the development of negative charge (or a decrease in the positive charge) on the amine nitrogen at the transition state, indicating the greater importance of a general base implicated in the deprotonation of the nucleophilic amine. A large absolute value for β_{nuc} is representative of an important effect at the transition state, whereas a shallow slope may be explained by two phenomena competing with each other at the transition state of a concerted reaction step, such as, for example, greater amine nucleophilicity *versus* increased ease of proton abstraction from the attacking amine. In the case presented here, the value determined ($\beta_{nuc} = 0.84$) is relatively large and positive, suggesting that the nucleophilic attack of the primary amine is more advanced than its concerted deprotonation at the transition state.

By way of contrast, the opposite situation has been observed for other transamidases. In particular, the subfamily EC 2.3.2 comprises enzymes that catalyze the transfer of the γ-glutamyl group to an amine acceptor substrate. Transglutaminase (TGase, EC 2.3.2.13) is another such enzyme that has been studied in our laboratory (*29*, *31*). We have shown that the deacylation step of its catalytic mechanism involves a concerted general base-catalyzed reaction, where the concomitant deprotonation of the primary amine acceptor substrate is more advanced at the transition state than its nucleophilic attack on the acyl−enzyme intermediate, giving a β_{nuc} of −0.37 (*29*). These studies have shown the importance of the conserved general base in the catalytic mechanism of TGase. For GGT, the mechanism could be quite different, for several reasons. The active site nucleophile of GGT may be a Thr hydroxyl group, rather than a Cys sulfhydryl group as in TGase, and the general acid/base residue of GGT may or may not be a His imidazole as has been suggested for TGase (*1*, *19*, *29*, *32*). For the serine protease chymotrypsin, similar structure−function studies of deacylation have shown that β_{nuc} is equal to ∼0 for aliphatic amines and ∼0.52 for aniline acceptor substrates (*33*). The pK_a values of the conjugate acids of the amines used in that study span a broad range, including the pK_a of the His responsible for deprotonation of the addition intermediate. The authors proposed that the rate-limiting step is the concerted (and efficient) proton abstraction during the nucleophilic attack of the amine substrate. These values demonstrate that even for similar enzymatic deacylation reactions featuring the same type of rate-limiting step, the efficiency of concomitant proton abstraction can have a profound effect on the sign and amplitude of the β_{nuc} values measured for the nucleophilic attack. In this way, β_{nuc} values are indicative of subtle geometric differences between the transition states of the different enzymatic reactions.

pH−Rate Profile Studies. A pH−rate profile was constructed for the substrate L-methionylpropylamide (**3d**) between pH 6.25 and 9.50 to determine the relative protonation states of the kinetically competent species. The rate-limiting step throughout the pH range that was studied was consistently the deacylation process, as evidenced by the absence of *y*-axis convergence of the reciprocal plots of kinetic data (see Experimental Procedures). As explained above, values of $k_{cat,b}^{norm}/K_b$ were used to study the pH dependence of the proposed nucleophilic attack of the acceptor substrate on the acyl−enzyme intermediate to give a tetrahedral intermediate. Several things are evident from the results shown in Figure 5. It is apparent that the curve is bell-shaped, featuring a broad plateau region between pH 7.0 and 9.0. This confirms that the Brønsted plot, done at pH 8.0, would probably be insensitive to small changes in pH. It is also important to note that while the data points at pH 6.25 and 9.50 contribute importantly to the overall shape of the curve, the enzymatic transpeptidation reaction is relatively inefficient at these pHs. Moreover, the kinetic analysis used herein requires significant reactivity differences between the competing hydrolysis and transpeptidation reactions at different acceptor substrate concentrations to provide precise values for the desired kinetic parameters (*16*). These factors contribute to the uncertainty in the values obtained at the extremes of the pH−rate profile, which is in turn reflected in the quantitative uncertainty in the pK_a values determined from the fitting of these results to a three-protonation state model (*34*). Nevertheless, it is evident that the ionization states of (at least) two functional groups are important during the overall process of the deacylation step catalyzed by GGT. The first ionization, with a kinetic pK_a of ∼7, could be due to the acceptor substrate. It has previously been shown that variation of the nature of the acceptor substrate affects the pK_a of the acidic ascending limb of pH−rate profile curves (*24*). However, it should be noted that the acidic pK_a that is determined does not correspond exactly to the pK_a (in solution) of the acceptor substrate used therein. The pK_a of the acceptor substrate used in our study, L-methionylpropylamide (**3d**), was determined by titration to be 7.40. Given the similarity of the first kinetic pK_a of Figure 5 with that of the acceptor substrate that was used, our results could be explained by the same theory advanced in the literature (*24*). On the other hand, it is also possible that the ionization of an essential general base could be hidden under the pK_a of the acceptor substrate such that the overlap of the two ionization curves hinders the clear identification of two similar but different pK_a values; however, the data do not permit the clear distinction of whether the slope has a value of one or two protons over this region. Moreover, the imprecision of the kinetic pK_a implies that a second pH−rate profile, obtained with an acceptor substrate having a very slightly different pK_a (≤0.3 unit), would not permit differentiation between these hypotheses. For the descending limb of Figure 5, the kinetic pK_a of ∼9 could be that of a general acid necessary to reprotonate the putative threonine active site nucleophile during the breakdown of the tetrahedral intermediate to regenerate the free enzyme.

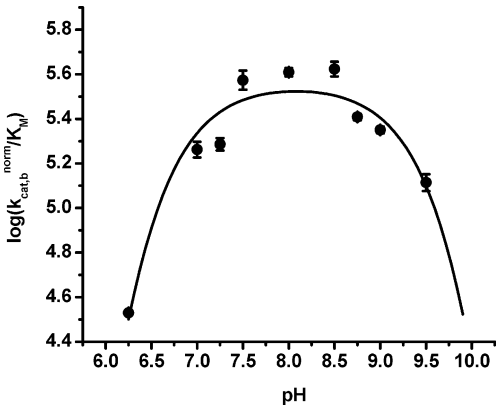
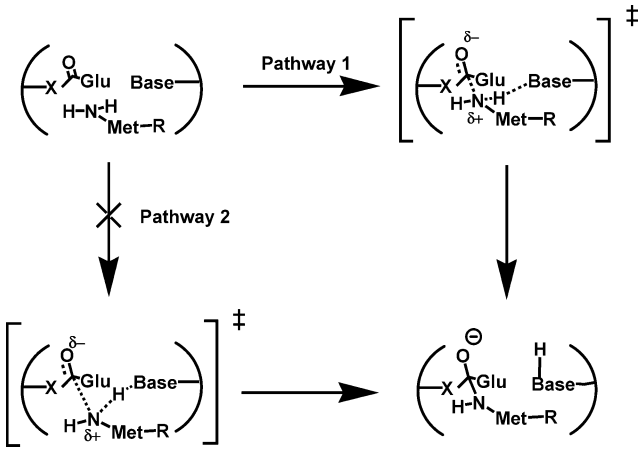


FIGURE 5: pH−rate profile plot of the transpeptidation reaction of rat kidney GGT with D-γ-glutamyl-*p*-nitroanilide as the donor substrate and L-methionylpropylamide (**3e**) as the acceptor substrate at 37 °C. For the protocol, see Experimental Procedures.

Solvent Isotope Effects. To determine the possibility of proton transfer at the transition state of the rate-limiting step of the deacylation process, solvent kinetic isotope effects were studied with three substrates: L-methionyl(2-fluoroethylamide) (**3c**), L-methionylpropylamide (**3d**), and L-methioninamide (**3f**). For each substrate, an isotope effect $(k_{cat,b}^{norm}/K_b)^{H_2O/D_2O}$ greater than unity was obtained. SKIEs of 1.77, 1.39, and 2.16 were calculated for the respective acceptor substrates. Interestingly, if the kinetic parameter of $k_{cat,b}^{norm}$ alone is considered, the corresponding SKIEs of $(k_{cat,b}^{norm})^{H_2O/D_2O}$ were calculated to be 2.53, 4.55, and 3.52,

Scheme 2



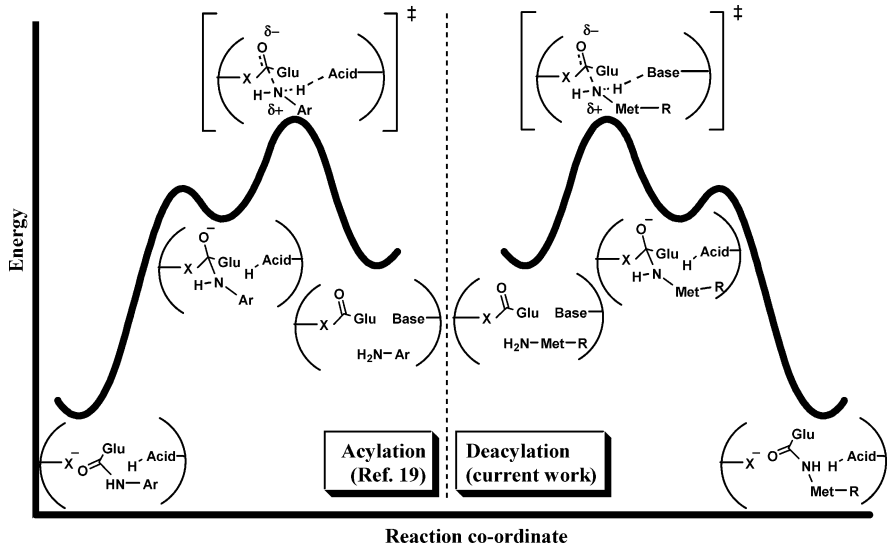
respectively. All of these values clearly suggest that there is a proton in flight at the transition state of the rate-limiting catalytic step for the deacylation process. Apparently, the small isotope effects observed for $k_{cat,b}^{norm}/K_b$ are due to significant decreases in both $k_{cat,b}^{norm}$ and K_b in D_2O relative to the values determined in H_2O. For example, in D_2O the measured K_b values drop to ~10−13 mM for each substrate, including L-methioninamide. The increased apparent affinity observed in D_2O compared to that in H_2O is well-known and probably due to a difference in solvation of the molecule in the reaction media (*35*, *36*). However, this change in solvent does not seem to affect the apparent affinity of all compounds equally, limiting the usefulness of the isotope effects determined for $k_{cat,b}^{norm}/K_b$, at least at a detailed quantitative level (*37*−*39*). Clearly, the most important point is that although there is no obvious correlation between the observed isotope effects and the acceptor substrate pK_a values, all of the measured isotope effects are greater than unity. This normal isotope effect is consistent with the transfer of a proton at the transition state of the rate-limiting step of the overall deacylation process.

On the basis of the above kinetic results from our studies of the linear free energy relationship, isotope effects, and the effect of pH, we propose, for the first time, a detailed mechanism for the deacylation step of GGT-mediated transpeptidation that is consistent with the experimental data, as shown in Scheme 2. In our proposed mechanism, the acyl−enzyme intermediate undergoes nucleophilic attack by the α-amino group of the L-methionine derivatives, concomitant with general base-assisted deprotonation. *A priori*, one could intuitively propose two types of transition states for this step. In pathway 1 of Scheme 2, the transition state features a short C−N partial bond and a short N−H partial bond, due to an advanced nucleophilic attack concomitant with an early transition state for the deprotonation of the amine. In the second proposed transition state (pathway 2 of Scheme 2), the C−N bond is extended, due to less advanced nucleophilic attack, and the N−H partial bond is very long since the proton is almost completely removed. Since more basic amines were shown herein to react more efficiently (considering the positive slope of the Brønsted correlation), it appears that the nucleophilic attack is more advanced at the transition state than the deprotonation of the amine of the acceptor substrate (pathway 1). The observed pH−rate profile and solvent kinetic isotope effects are also consistent with this proposed mechanism. At low pH values, the amine is protonated as its ammonium group, decreasing the concentration of active species capable of nucleophilic attack on the acyl−enzyme intermediate. The pK_a of a putative general base capable of concerted deprotonation of the acceptor substrate amine could not be determined, and may be obstructed by the ionization of the acceptor substrate. The requirement of an acidic residue in the deacylation reaction, evident in the basic limb of the pH−rate profile, may point to the necessity of reprotonation of the putative active site threonine residue during decomposition of the tetrahedral intermediate. Finally, the proton in flight at the rate-limiting transition state of our proposed mechanism (Scheme 2) is consistent with our observed SKIE data.

It is instructive to compare the results presented herein for the deacylation step with those reported for the acylation step (*19*). In our previous report, L-γ-glutamyl anilide derivatives containing electron-donating and electron-withdrawing groups were used as acyl−donor substrates under conditions in which acylation was rate-limiting. The Hammett plot obtained in that study, along with other kinetic data, allowed us to propose a mechanism for acylation where the rate-limiting step is the decomposition of a tetrahedral intermediate to give the acyl−enzyme intermediate and liberate the aniline leaving group. For the most basic anilines (shown to display a β_{lg} value of 0.43), whose basicity is comparable to that of L-Met derivative acceptor substrates, we proposed that the transition state of the rate-limiting step features significant protonation of the leaving group amine and less advanced cleavage of the C−N bond (Acylation, Scheme 3). According to the law of microscopic reversibility (*30*), the reverse reaction—namely, aminolysis of the acyl enzyme to give a secondary amide product, similar to the transpeptidation reaction studied herein—should proceed via the same mechanism, where the transition state involves significant nucleophilic attack concurrent with less advanced deprotonation (Deacylation, Scheme 3). This corresponds exactly to the mechanism proposed herein based on current kinetic studies of the deacylation step.

Previous studies have been performed to attempt to identify the amino acids implicated in the acylation and deacylation steps of the catalytic cycle of GGT. Initially, Ser451 and Ser452 were identified by mutagenesis studies as being potential active site nucleophiles of human GGT (*40*). However, for *Escherichia coli* GGT, the N-terminal threonine of the light subunit, which is also conserved among all mammalian GGTs, has been identified as the active site nucleophile by irreversible inhibition and mass spectrometry. According to these results, GGT could be classified as a probable member of the family of the Ntn-hydrolases (*41*). With respect to interactions with the acceptor substrate during the deacylation step, an arginine residue of bovine milk GGT has been identified through chemical modification with butanedione (*42*). It was proposed that this Arg is implicated in acceptor substrate binding by dipole−cation interaction with the carbonyl group of the acceptor substrate, since only transamidation, and not hydrolysis, was affected by its modification. This result was corroborated by site-directed mutagenesis experiments, in which the replacement of the analogous Arg107 of human GGT with a lysine residue had a significant effect on acceptor substrate binding affinity (*43*). In the same study, the Glu108 residue was mutated to Gln.

Scheme 3



The resulting loss of charge on residue 108 was also found to have a profound effect on substrate binding and catalysis in the deacylation step. Thus, it is possible that Glu108 is responsible for the deprotonation of the amine acceptor substrate. These mutagenesis studies are consistent with our results, but no confirmation of the exact role of these amino acids has been possible without knowledge of the three-dimensional structure of the enzyme. To date, the only three-dimensional structure known for GGT is that of the *E. coli* enzyme, which has never been resolved to sufficiently high resolution to permit the unambiguous identification of active site residues (*44*, *45*).

Prior to any discussion of the physiological roles that GGT may play, it is instructive to consider the scope and limitations of the transformations that it catalyzes. GGT is known to display relatively strict specificity toward its acceptor substrates, recognizing predominantly amino acids with the L-configuration. Although it has been reported that primary amines can serve as acceptor substrates, (*2*, *16*), we have found that in general they do not react efficiently and are not recognized well by rat kidney GGT (*46*). It has also been postulated that GGT may exhibit slow esterase activity (*16*, *47*), suggesting that, according to the law of microscopic reversibility, alcohols may also function as acceptor substrates. However, we have found that most alcohols are not recognized as such by GGT, although L-lactic acid was found to be a weak (millimolar) competitive inhibitor of glycylglycine as an acceptor substrate (*46*). Given the strict specificity that GGT displays for amino acid acceptor substrates having the L-configuration, it is possible that L-lactic acid, representing the alcohol derivative of alanine, is able to bind in the acceptor binding site since it bears a carboxylate carbonyl group necessary for recognition as an acceptor substrate. However, its hydroxyl group appears to be too unreactive to form a new ester bond upon reaction with the acyl−enzyme intermediate. One may postulate that the hydroxyl group oxygen is less nucleophilic than the α-amino group of L-amino acids such that the deacylation process cannot occur. We are currently developing an HPLC-based method for detection of acyl transfer reaction products, to gain further insight into the scope of GGT reactivity with alcohols as well as with thiols.

While hydrolysis and transamidation reactions of glutathione derivatives remain the reactions most efficiently catalyzed by GGT, the exact physiological role of the enzyme is still unknown. Some authors have shown that at pH 7.4, the γ-glutamyl acyl−enzyme intermediate could react equally well with water or amines, because of the high physiological concentration of α-amino acids (3.11 mM) in the proximity of GGT (*17*). At pH 8.0, deacylation was proposed to be the preferred reaction (*17*). Some insight into this discussion is also provided by our current results, namely, the partition coefficient (K_{iab}) calculated for each acceptor substrate studied herein. This partition coefficient signifies the concentration of the α-amino acid nucleophile necessary for the transamidation reaction to proceed at the same rate as the hydrolysis reaction during catalytic deacylation (*48*). For the L-methionine amides that have been studied, we have found partition coefficients between 1.21 mM [for L-methioninamide (**3f**)] and 4.99 mM [for L-methionyl(2,2,2-trifluoroethylamide) (**3a**)]. In general, the calculated partition coefficient is smallest for acceptor substrates with the highest pK_a, closest to the pK_a values of typical physiological α-amino acids and dipeptides (∼9.2). Using the estimated physiological concentration of α-amino acids of 3.11 mM, and projected partition coefficients of less than 1.21 mM, one could infer that under physiological conditions, transpeptidation (or aminolytic deacylation) would be the principal reaction catalyzed by GGT.

In conclusion, we have proposed a mechanism for the deacylation step of GGT-mediated transpeptidation using L-methionine derivatives as acceptor substrates. This mechanism is consistent with the observed Brønsted linear free energy relationship, pH−rate profile, and solvent kinetic isotope effects. It is proposed that in the rate-limiting step these acceptor substrates carry out a nucleophilic attack on the acyl−enzyme intermediate, with simultaneous deprotonation of the nucleophilic amino group, to form a tetrahedral intermediate. At the transition state, nucleophilic attack is more advanced than deprotonation of the amine, leading to significant development of positive charge on the nucleophilic nitrogen. Further studies of the reactivity and structural nature of the acyl−enzyme intermediate are currently underway in our laboratory.

SUPPORTING INFORMATION AVAILABLE

Experimental protocol for the synthesis of compounds **3a**−**3e** and their characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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