Kinetic Studies of Guinea Pig Liver Transglutaminase Reveal a General-Base-Catalyzed Deacylation Mechanism†

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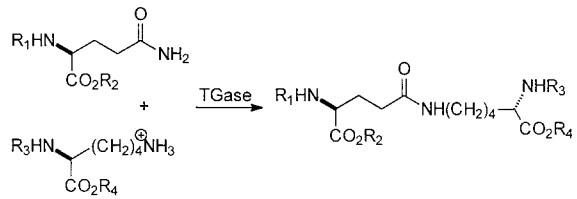
ABSTRACT: Guinea pig liver transglutaminase (TGase) reacts with 0.1 mM *N*-Cbz-L-Glu(γ-*p*-nitrophenyl ester)Gly (**5**, prepared herein, K_M = 0.02 mM) to undergo rapid acylation that can be followed spectrophotometrically at 400 nm (pH 7.0, 25 °C). Deacylation of the transiently formed thiolester acyl enzyme intermediate via catalytic aminolysis was studied in the presence of six primary amines of widely varying basicity (pK_{NH+} = 5.6−10.5). Steady-state kinetic studies were performed to measure k_{cat} and K_M values for each amine substrate. A Brønsted plot constructed through the correlation of log(k_{cat}/K_M) and pK_{NH+} for each amine substrate displays a linear free-energy relationship with a slope $\beta_{nuc} = -0.37 \pm 0.08$. The shallow negative slope is consistent with a general-base-catalyzed deacylation mechanism in which a proton is removed from the amine substrate during its rate-limiting nucleophilic attack on the thiolester carbonyl. Kinetic isotope effects were measured for four acceptor substrates (water, kie = 1.1 ± 0.1; aminoacetonitrile, kie = 5.9 ± 1.2; glycine methyl ester, kie = 3.4 ± 0.7; *N*-Ac-L-lysine methyl ester, kie = 1.1 ± 0.1) and are consistent with a proton in flight at the rate-limiting transition state. The active site general-base implicated by these kinetic results is believed to be His-334, of the highly conserved TGase Cys-His-Asp catalytic triad.

Transglutaminases (TGases,[1] EC 2.3.2.13) are enzymes found in all vertebrates that belong to a class of enzymes known as aminoacyltransferases, which modify peptides and proteins by catalyzing the formation of isopeptide cross-links via acyl transfer reactions. Their calcium-dependent catalytic activity is exhibited toward γ-carboxamide groups of peptide-bound glutamine residues, which serve as acyl donors to ϵ-amino groups of peptide-bound lysine acceptor residues, resulting in new intermolecular ϵ−(γ-glutamyl)lysine cross-links (*1*) (eq 1). The resulting covalent amidic bonds are stable and resistant to chemical, enzymatic, and physical degradation.



(1)

In mammals, TGases are distributed throughout the plasma, tissues, and extracellular fluids, and comprise a large family of proteins. They are distinguishable from each other on the basis of their physical properties as well as their distribution in the body. Six distinct TGases have been isolated and characterized to date. One well-known member is the blood clotting enzyme Factor XIIIa, which is localized in plasma and impedes blood loss by stabilizing fibrin clots during their formation at sites of blood coagulation. We have focused our attention on the ubiquitous "soluble" or "tissue" protein known as TGase2 (*2*, *3*). Tissue transglutaminase is a monomeric protein containing 690 amino acid residues, has a molecular mass of 85 kDa, and is neither glycosylated nor disulfide bonded (*1*). Various cell types express tissue TGase such as liver, kidney, lung, intestine, spleen, brain, and endothelial cells as well as smooth muscle cells of arteries, veins, and capillaries (*1*). The enzyme is largely intracellular, but may also be localized in the interstitial fluids. Although there is no consensus yet regarding its precise physiological role, tissue TGase has been ascribed to several processes, including stabilization of extracellular matrixes, formation of cross-linked cell envelopes, cell-matrix assembly, wound healing, and cellular adhesive processes (*1*).

Tissue TGase has been implicated in many physiological disorders characterized by increased concentrations of cross-linked proteins. These include the onset of thrombosis and atherosclerosis (*4*), deposition of insoluble amyloid plaques in the brain leading to Alzheimer's disease (*5*), wheat gliadin-induced intestinal inflammatory disease known as celiac disease (*6*), cataract development due to βA3-crystallin deposition on the eye lens (*7*), premature red blood cell aging and elimination from circulation known as Hb-Koln disease (*8*), and neurodegenerative diseases (such as Huntington's

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[1] Abbreviations: TGase, transglutaminase; bs, broad singlet; Cbz, benzyloxycarbonyl; d, doublet; dd, doublet of doublets; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; 2,4-DNP, 2,4-dinitrophenyl acetate; kie, kinetic isotope effect; m, multiplet; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pNPA, *p*-nitrophenyl acetate; s, singlet; t, triplet; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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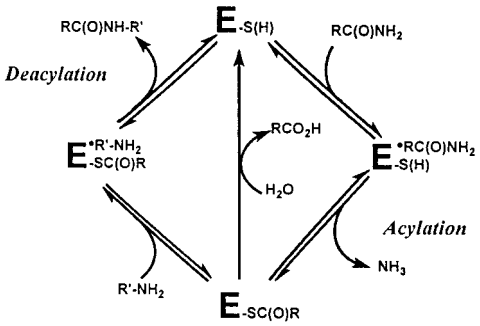
Scheme 1



disease) associated with an increase in polyglutamine-containing peptides in the brain (*9*). Due to its broad involvement in disease, tissue TGase is a compelling candidate for therapeutic intervention. A viable approach to treatment is the use of mechanism-based inhibitors, which provides incentive for detailed mechanistic studies of the enzyme.

Guinea pig liver, because of its endogenous rich abundance of TGase in comparison to rat, mouse, rabbit, and calf liver, has become the mostly widely cited source of TGase for research (*10*), and several protocols have been published for its purification (*11*−*14*). Furthermore, guinea pig liver TGase shows 80% homology with human tissue TGase, thereby validating its use as a model for the elucidation of the general catalytic mechanism by which transglutaminases form ϵ−(γ-glutamyl)lysine cross-links (*1*). Finally, TGase from guinea pig liver has a much broader substrate specificity, making it suitable for the substrate analogue kinetic studies described herein.

The reaction catalyzed by TGase is known to proceed by the modified "ping-pong" mechanism shown in Scheme 1 (*15*). An active site thiol (cysteine residue) is transiently acylated by the γ-carboxamide group of peptide-bound glutamine (acyl donor) with the release of ammonia. The acylation step is an energetically uphill reaction, which results in the formation of a thiolester acyl enzyme intermediate. The driving force for the reaction is partially supplied by the release of ammonia and its subsequent protonation to ammonium occurring readily at physiological pH (*1*). Deacylation occurs through either hydrolysis or aminolysis of the intermediate thiolester acyl enzyme, resulting in the regeneration of free enzyme. The acyl acceptor during this process is typically either the ϵ-amino group of a peptide-bound lysine or the primary amino group of some naturally occurring polyamine (such as spermidine or putrescine). In the absence of a primary amine acceptor, water is capable of regenerating free enzyme, albeit much more slowly.

Transglutaminases have demonstrated in vitro catalytic activity toward certain active esters, catalyzing their hydrolysis and aminolysis (*16*). TGase catalytic activity is most likely effected by a conserved active site Cys-His-Asp triad reminiscent of that of cysteine proteases. This triad has recently been identified by X-ray crystallography as Cys-314, His-373, and Asp-396 (*17*) in Factor XIIIa, the only TGase which has been crystallized and for which a three-dimensional structure is available. In the crystal, the sulfur atom of Cys-314 is within 3.2 Å of the $N^{\delta 1}$ atom of the imidazole ring of His-373, suggesting the presence of an imidazolium−thiolate ion pair, which is also featured in the case of the cysteine proteases (*18*). Comparison of the amino acid sequences of Factor XIIIa and guinea pig liver TGase indicates this presumed catalytic triad would correspond to Cys-276, His-334, and Asp-358 in the active site of guinea pig liver TGase (*19*) by virtue of the highly conserved sequence in the active site region of all TGases.

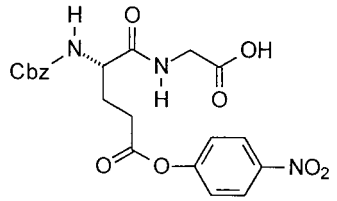


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FIGURE 1: Acyl donor substrate analogue *N*-Cbz-Glu(γ-*p*-nitrophenyl ester)Gly (**5**). See Supporting Information for synthesis details.

To investigate the general mechanism of TGase catalysis, we have prepared a high-affinity active ester substrate analogue and pursued detailed kinetic studies using a series of primary amines of varying basicity as acyl acceptor substrates. Through the application of Brønsted catalysis theory, we have probed the role of a putative active site general-base during the rate-limiting deacylation step of the enzymatic transamidation reaction. Our results are reported herein.

MATERIALS AND METHODS

Materials. Bio-Rad D_C Protein Assay Solution, Macro-Prep DEAE Support, and Bio-Gel A-0.5m Gel were obtained from Bio-Rad. BSA (bovine serum albumin, Fraction V) was purchased from Sigma-Aldrich. *N*-Cbz-L-GlnGly was synthesized in our laboratory according to a published procedure (*11*). All aqueous solutions were prepared using deionized water purified with the Millipore BioCell system.

Substrate Analogue Synthesis. The active ester **5** (Figure 1) was synthesized as an acyl-donor substrate analogue, according to the detailed procedures found in the Supporting Information section.

Enzyme Purification. Guinea pig livers were obtained fresh on ice either from Rockland, Inc. (Gilbertsville, PA), or as a generous gift from Dr. S. Rowland of Merck-Frosst (Pointe-Claire/Dorval, PQ). TGase was isolated from the livers and purified according to a protocol recently developed in this laboratory (*14*), based on the modification of previously published procedures (*11*, *12*, *23*). This modified protocol provides guinea pig liver TGase in greater yield and activity than previously available.

Methods. Concentrations of total protein were determined using the Bio-Rad D_C Protein Assay, a method based on the Lowry assay (*24*) (using BSA as a standard) at all stages of the purification except for fractions obtained after size exclusion chromatography. For these fractions, TGase was considered to be of high purity, so that protein concentrations were determined using the extinction coefficient for TGase ($A^{1\%}_{280nm} = 15.8$) (*11*). All spectrophotometric measurements were made using a temperature-controlled Varian Cary 100 UV−vis spectrophotometer.

TGase activity was measured by the colorimetric hydroxamate assay procedure using 30 mM *N*-Cbz-L-GlnGly, 1 mM EDTA, 5 mM $CaCl_2$, and 0.1 M hydroxylamine in 0.2 M Tris−acetate at pH 6.0 and 37 °C (*12*). The activity assay

solution was prepared fresh daily. One unit of enzyme activity (U) is defined as the amount of TGase required to catalyze the formation of 1 μmol of γ-glutamylhydroxamic acid per minute. Specific activity is reported as units of activity (U) per milligram of protein.

Enzyme Reaction Product Extinction Coefficients. The progress of the reaction of TGase with substrate analogues *p*-nitrophenyl acetate (pNPA), 2,4-dinitrophenyl acetate (2,4-DNP), and *N*-Cbz-L-Glu(*p*-nitrophenyl ester)Gly (**5**) was monitored as an increase of absorbance at 400 nm due to the release of either *p*-nitrophenolate or 2,4-dinitrophenolate. Primary kinetic data were obtained as slopes of absorbance versus time, which were transformed into reaction rates by dividing by the appropriate extinction coefficient. These extinction coefficients were measured from solutions that were prepared to closely resemble those used in the kinetic experiments and included 5% DMF, 50 mM $CaCl_2$, 50 μM EDTA, 0.1 M MOPS (pH 7.0). Standard curves were constructed (in duplicate) by measuring the absorbance values at 400 nm and 25 °C of eight solutions of *p*-nitrophenol (10−120 μM) or 2,4-dinitrophenol (0.25−5.0 μM). The slopes of this standard curves gave the required extinction coefficients of 8040 M^{-1} cm^{-1} for *p*-nitrophenolate and 12 600 M^{-1} cm^{-1} for 2,4-dinitrophenolate.

Steady-State Kinetics. Steady-state kinetic assays of TGase-mediated reactions were performed in 0.1 M MOPS buffer (pH 7.0) that included 50 μM EDTA, 50 mM $CaCl_2$, and 5% DMF at 25 °C. To study the catalyzed hydrolysis reaction, at least 10 different concentrations of either *N*-Cbz-L-glutamyl(γ-*p*-nitrophenyl ester)glycine **5** (0.001−0.8 mM), or 2,4-dinitrophenyl acetate (0.01−2.5 mM), or *p*-nitrophenyl acetate (0.05−4.0 mM) were present as acyl donor substrates. Reactions were initiated by the addition of 10−15 μL of purified TGase (0.7−1.0 mg/mL) and were followed as a linear increase in absorbance at 400 nm over a maximum of 10 min after the initial "burst". Reaction solution pH was measured before and after the runs and found to vary by less than 0.1 unit. Slopes of absorbance vs time were transformed into reaction rates by dividing by the appropriate extinction coefficient (vide supra). Blanks containing all components of the kinetic assay solution except enzyme were conducted in duplicate and used directly to correct the observed catalytic reaction rates. Initial velocities obtained at different acyl donor concentrations were corrected for the mass of enzyme present, normalized (to 14 U/mg) according to the activity of the enzyme utilized, and used to construct a hyperbolic Michaelis−Menten saturation curve. The kinetic parameters of V_{max} and K_M were obtained through nonlinear regression of these data according to eq 2 using Axum 5.0 curve-fitting software.

$$v = \frac{V_{max}[sub]}{K_m + [sub]} \quad (2)$$

To study the catalyzed aminolysis (transamidation) reaction, steady-state kinetic assays were performed as described above using 0.1 mM *N*-Cbz-L-glutamyl(γ-*p*-nitrophenyl ester)glycine (**5**) as an acyl donor substrate, and at least 10 different concentrations each of a series of primary amines as acyl acceptor substrates. The ranges of acyl acceptor substrate concentrations employed were the following: aminoacetonitrile (0.02−3.0 mM), 2,2,2-trifluoroethylamine (0.1−15 mM), glycine methyl ester (0.1−12 mM), glycinamide (0.2−18 mM), 2-(ethylthio)ethylamine (0.5−40 mM), and *N*-acetyl-L-lysine methyl ester (1.0−40 mM). Reactions were initiated by the addition of substrate **5** and followed over the first 5 min after the initial "burst". Blanks contained all components of the kinetic assay solution except enzyme. Initial velocities, measured in duplicate for each primary amine concentration, were used to construct a hyperbolic Michaelis−Menten saturation curve. The maximal rates observed in the presence of primary amine acceptor substrates were always higher than the background catalytic hydrolysis reaction rate (V_{hyd}^{max}); however, at very low amine concentrations, V_{hyd}^{max} becomes relatively significant. The apparent kinetic parameters for transamidation were therefore determined by nonlinear regression according to the slightly modified kinetic model given below as eq 3.

$$v = \frac{V_{max}[sub]}{K_m + [sub]} + V_{hyd}^{max} \quad (3)$$

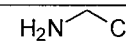
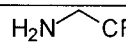
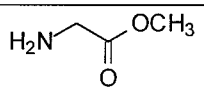
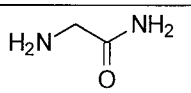
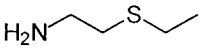
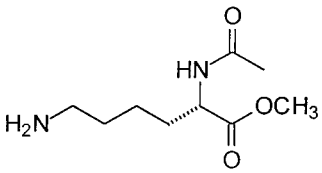
Values of V_{max} were subsequently converted into k_{cat} values by dividing by the concentration of enzyme, using a molecular mass of 85 kDa (*14*).

Titrations. To determine the pK_a of the conjugate acid (ammonium ion) of each primary amine studied, 0.2 M solutions of primary ammonium chlorides were prepared containing 5% DMF where the ionic strength was adjusted to 0.25 M with KCl. Pretiter volumes of 1 mL of 0.1 N HCl were added when necessary to lower the initial sample pH. The ammonium ion solutions were then titrated with 0.1 N NaOH. Titrations were performed at least in duplicate using a Mettler Toledo DL53 autotitrator. The primary alkylammonium pK_a values were calculated as the pH of the titration solution halfway to the measured equivalence point and are reported in Table 1.

pH Studies. The effect of pH on the kinetic parameters of the enzymatic transamidation reaction was investigated by following the procedure described above using 0.1 M buffer solution to maintain pH values at 6.0, 6.5 (MES), 7.0, 7.5 (MOPS), and 8.0 (Tris-HCl). The stability of TGase at the various pH levels studied was confirmed by incubating the enzyme at 25 °C in various buffers over a time period representative of a typical kinetic run (<5 min), followed by verification of its activity using the standard hydroxamate activity assay (*12*). Over the pH range studied (pH 6−8), activity was found to vary by less than 5%. Extinction coefficients for *p*-nitrophenolate at each pH were determined to be 1750 M^{-1} cm^{-1} (pH 6.0), 4100 M^{-1} cm^{-1} (pH 6.5), 8040 M^{-1} cm^{-1} (pH 7.0), 13 700 M^{-1} cm^{-1} (pH 7.5), and 18 050 M^{-1} cm^{-1} (pH 8.0).

Isotope Effect Studies. To measure the isotope effect on the apparent transamidation reaction rate, the same procedure was used as reported above for the steady-state kinetic studies, except that all solutions used in the reaction mixtures were prepared with D_2O. Reaction rates were followed for at least 10 concentrations of aminoacetonitrile(0.01−2.0 mM), glycine methyl ester (0.1−12 mM), and *N*-acetyl-L-lysine methyl ester (1−10 mM). The pD values of the deuterated solutions were calculated by adding 0.4 to the values reported by the Fisher Accumet 15 pH meter equipped with a calomel electrode (*25*).

Table 1: Kinetic Parameters [k_{cat}, K_M, and log(k_{cat}/K_M)] Obtained According to Equations 2 and 3 for the Reaction of TGase at 25 °C and pH 7.0 (0.1 M MOPS) with 0.1 mM **5** in the Presence of a Series of Primary Amine Acceptor Substrates, and the pK_a Values of Their Respective Conjugate Acid Ammonium Ions

Acceptor substrate	Structure	k_{cat} [a]	K_M [b]	log(k_{cat}/K_M) [c]	Ammonium pK_a [d]
Aminoacetonitrile		114	0.098	3.06	5.64
2,2,2-Trifluoroethylamine		62.2	0.492	2.10	6.02
Glycine methyl ester		71.4	0.626	2.06	7.92
Glycinamide		105	4.68	1.35	8.23
2-(Ethylthio)ethylamine		74.7	5.88	1.10	9.57
N-Acetyl-L-lysine methyl ester		28.3	2.80	1.00	10.50

[a] Given in units of min^{-1} and normalized for a specific activity of 14 U/mg; experimental error is estimated at ±10%. [b] Given in units of mM; experimental error is estimated at ±10%. [c] Experimental error is estimated at ±0.05. [d] Measured titrimetrically in 5% DMF and 0.25 M KCl at 25 °C (see Methods). Experimental error is estimated at ±0.02 unit.

RESULTS AND DISCUSSION

Synthesis of Active Ester Substrate Analogue ***5****.* *N*-Cbz-L-Glutamyl(*γ*-*p*-nitrophenyl ester)glycine (**5**, Figure 1) was prepared for use as a substrate analogue for the kinetic study of TGase deacylation because it satisfied certain essential criteria. Namely, the peptide framework is known to confer good affinity for the TGase binding site (*26*), the active ester moiety is capable of rapidly acylating the enzyme (*27*), making the deacylation step rate-limiting (*22*) (vide infra), and the reaction product released upon acylation of the enzyme (*p*-nitrophenolate) is an excellent chromophore, permitting the enzymatic reaction progress to be followed spectrophotometrically. Although a synthetic route to this compound was published in 1970 (*11*), several different synthetic routes to **5** were developed in this laboratory (*28*) in order to optimize the yield and practicality of its preparation, while minimizing the degree of possible racemization, since the D-isomer has a markedly higher K_M value (*22*). The details of our preferred synthetic route are included in the Supporting Information section.

The usefulness of substrate analogue **5** is particularly apparent considering the advantages of its employment in kinetic studies. The spectrophotometric kinetic assay of the reaction of **5** is rapid, direct, continuous, and sensitive. Unfortunately, it is also practically limited to use around neutrality. At low pH, the method loses its sensitivity as the bright yellow *p*-nitrophenolate chromophore is protonated in solution to its less brightly colored conjugate acid [*p*-nitrophenol pK_a = 7.16 (*33*)]. At high pH, active ester **5** is susceptible to rapid nonenzymatic background hydrolysis. Nevertheless, for the kinetic studies at pH 7.0 described herein, the substrate analogue was especially appropriate. As an active ester, it is capable of rapidly acylating the enzyme, making the subsequent steps of the catalytic cycle rate-limiting and kinetically prominent. Furthermore, as a derivative of Cbz-L-Glu-Gly, it forms the same acyl enzyme intermediate as would Cbz-L-Gln-Gly, a widely used substrate that resembles the native substrate in which the donor glutamine residue is peptide-bound. This allows for comparison of kinetic data and constitutes the relevance of our mechanistic conclusions to the biological system.

Rate-Limiting Deacylation. The TGase-catalyzed hydrolysis of substrate analogue **5** was studied under steady-state conditions at pH 7.0 and 25 °C as described above (see Materials and Methods). The kinetic parameters obtained according to eq 2 are V_{max} = 0.20 mM min^{-1} (mg of enzyme)$^{-1}$ (k_{cat} = 17.0 min^{-1}) and K_M = 0.02 mM for **5** (for the rate-limiting hydrolysis reaction). These values are similar to those obtained earlier by Folk (*22*) [V_a = 50 μmol min^{-1} (μmol of enzyme)$^{-1}$ for hydrolysis and K_M = 0.009 mM]. For subsequent kinetic studies performed in the presence of primary amine acceptor substrates, it was important to choose reaction conditions that would ensure rapid acylation, so that the results of the ensuing kinetic studies could be interpreted as being pertinent to rate-limiting deacylation. These reaction conditions include a concentration of substrate analogue **5** of 0.1 mM, which is 5 times the value of the apparent K_M.

The rapid acylation of TGase by active ester substrate analogue **5** (followed by rate-limiting deacylation) is clearly demonstrated by the observed "burst" kinetics. Quantitative analysis of the "burst" phase could yield rate constants for the acylation step, but requires stopped-flow mixing experiments, and will be reported elsewhere. For the purposes of the present study, our preliminary examination of the burst verified that it was approximately proportional to the concentration of enzyme present and relatively constant under the kinetic conditions used herein to study the rate-limiting deacylation step represented by the linear "slow" phase (*28*).

Comparison of the literature turnover rates for the hydrolysis of **5** (*22*, *28*) and of Cbz-Gln-Gly (*26*, *34*), a popular amide substrate, reveals that the overall rates for these reactions (involving the same acyl enzyme intermediate) are in fact very similar (17 vs 20 min^{-1}, respectively). This suggests that in the case of hydrolysis, the rate-limiting step of the catalytic cycle is deacylation, even following acylation by an amide substrate. Rapid acylation from Cbz-Gln-Gly is also evident from the tremendous acceleration (~100-fold) of the overall turnover rate that is realized upon changing the acceptor substrate from water, in the case of hydrolysis, to hydroxylamine, as employed in the standard activity assay reaction (~1400 min^{-1}) (*12*). If acylation by Cbz-Gln-Gly were rate-limiting, then changing the acyl acceptor substrate from water to hydroxylamine would not result in an increased reaction rate. However, rapid overall reaction rates with Cbz-Gln-Gly have been observed (*22*, *26*, *28*, *31*, *34*) that vary according to the identity of the acceptor substrate, demonstrating that acylation, even from an amide, is not rate-limiting. In the present study, an active ester substrate (**5**) is used to acylate the enzyme, presumably even more rapidly, and the overall reaction rate was found to vary according to the acyl acceptor substrate used, again implying rate-limiting deacylation.

By way of confirmation, the TGase-catalyzed hydrolyses of *p*-nitrophenyl acetate (pNPA) and 2,4-dinitrophenyl acetate (DNPA) were also studied. These active esters release different leaving groups on forming identical transient acetyl enzymes. The turnover numbers measured for these hydrolysis reactions were found to be identical within experimental error (45 ± 12 min^{-1}), indicating that in both cases hydrolytic deacylation is rate-limiting. Since substrate analogue **5** has the same leaving active ester moiety as pNPA, we may infer that for the hydrolysis and aminolysis reactions of **5** studied herein, acylation from **5** is also rapid. This signifies that the following kinetic studies provide information relevant to the mechanism of rate-limiting deacylation.

Brønsted Correlation for the Deacylation Step. Several studies performed to map out the specificity of guinea pig liver TGase for its acyl acceptor substrate (*26*) have shown that the enzyme demonstrates a rather broad substrate specificity, and is capable of catalyzing γ-glutamyl acyl transfer to many different primary amines. The present study takes advantage of this wide substrate recognition to explore the mechanistic impact of using six primary amines of slightly varying structure and widely varying basicity.

Using substrate analogue **5** as an acyl donor, steady-state kinetic studies were carried out at pH 7.0 as described above, in the presence of six different primary amines whose conjugate acid pK_a values ranged from pK_{NH+} 5.6 to 10.5. Correlation of the log(k_{cat}/K_M) values obtained for these transamidation reactions with the pK_{NH+} values of the corresponding amines was found to display a linear free-energy relationship, as shown in Figure 2 (and Table 1). This Brønsted plot features a shallow negative slope ($\beta_{nuc} = -0.37 \pm 0.08$) with good linearity over the broad range of amine basicities studied, indicating there is no change in the rate-limiting step, confirming that in all cases, deacylation is the rate-limiting catalytic process. In contrast, the plot of log(k_{cat}) values against pK_{NH+} for the amines studied was extremely scattered (results not shown). The fact that it is necessary to implicate the K_M values of the amines studied in order to observe a linear free-energy relationship signifies the rate constants for equilibrium substrate binding are incorporated in the rate law for the rate-limiting step for the enzymatic deacylation process. This suggests that the rate-limiting step in deacylation is a function of the events leading to the formation of the presumed covalent (tetrahedral) intermediate, and not the decomposition of this intermediate. The rate-limiting step is therefore likely to be a covalent bond formation event between the substrate and the enzyme (such as the nucleophilic attack of the amine substrate on the acyl enzyme thiolester) which takes into account the rapid equilibrium binding of the substrate.

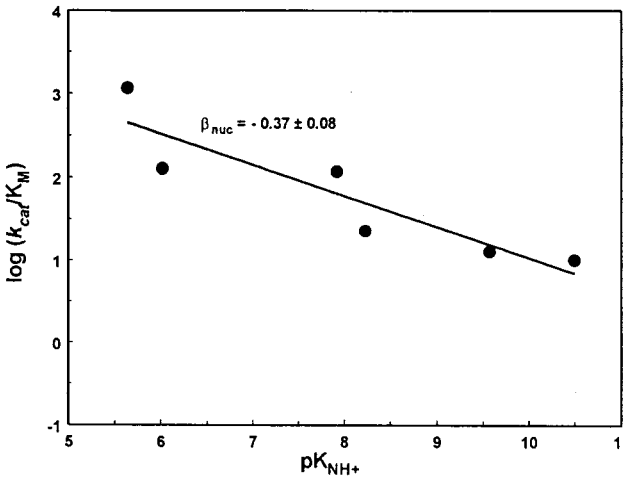
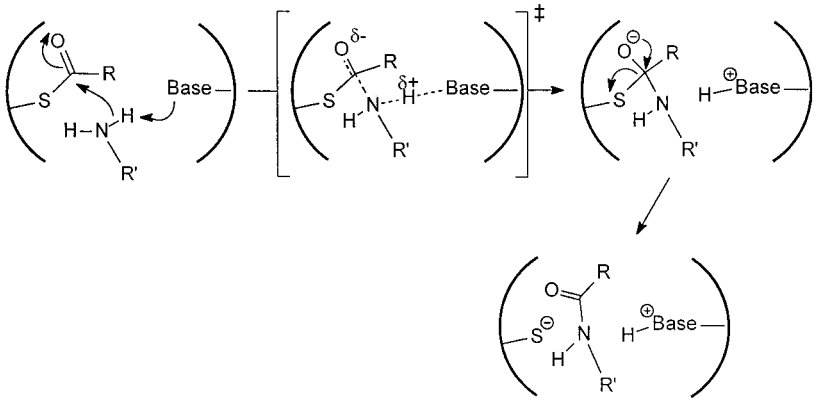


FIGURE 2: Brønsted plot of the kinetic data according to eq 3 for the reaction of guinea pig liver TGase, in the presence of 0.1 mM *N*-Cbz-Glu(γ-*p*-nitrophenyl ester)Gly (**5**), with a series of primary amine acceptor substrates at 25 °C and pH 7.0 (0.1 M MOPS) (see Table 1 and Materials and Methods). Linear regression of the data points resulted in the line through the data of which the slope $\beta_{nuc} = -0.37 \pm 0.08$.

In general, a positive Brønsted β_{nuc} parameter for the aminolysis reaction would have indicated the development of positive charge on the nitrogen at the transition state, and that amine nucleophilicity is important in the rate-limiting step of the deacylation process. Conversely, a negative slope would signify that negative charge is developing (or positive charge is decreasing) on the nitrogen at the transition state, as would be the case if proton transfer from the substrate amine were involved, the reaction being faster with more acidic amines. Furthermore, the relative size of the β_{nuc} parameter indicates the degree of the combination of the formation of the corresponding bonds at the rate-limiting transition state. The fact that the β_{nuc} slope of Figure 2 is negative indicates that the rate-limiting step of aminolysis of the intermediate TGase thiolester is not as influenced by the nucleophilicity of a primary amine, as much as by its relative acidity. This phenomenon is consistent with a mechanism involving proton transfer from the attacking amine during the rate-limiting step, as would be anticipated in the case of general-base-catalyzed aminolysis, where proton transfer occurs simultaneously with nucleophilic attack upon the intermediate thiolester carbonyl (Scheme 2). The shallowness of the slope of Figure 2 probably reflects the net result of two competing effects prevailing from the concerted reaction pathway, where the greater nucleophilicity of the more basic amines is offset by the increased ease of proton abstraction from amines of higher acidity. In contrast,

Scheme 2



the nonenzymatic aminolysis of thiolesters such as *p*-nitrophenyl thiolacetate is known to proceed by a stepwise mechanism, where the rate-limiting step involves only nucleophilic attack, giving rise to a large positive slope, $\beta_{nuc} = +0.83$ (*35*).

Given that the native substrate of TGase is a peptide-bound lysine, it is apparent that at physiological pH, about 99.9% of the ϵ-amino group of the native substrate in solution will be present in its protonated state. Although the reactive nucleophilic form of the substrate must be the neutral amine, it seems reasonable to assume that TGases are capable of recognizing and binding primary amine substrates in both protonation states. The proton on the lysine ϵ-ammonium group may be lost to bulk solvent upon binding, just as, in the microscopic reverse reaction, ammonia formed during acylation by primary amides would be protonated by bulk solvent upon its release (*34*). For this reason, it was deemed unnecessary to adjust the concentrations of the primary amine substrates used to correct for one particular state of ionization. If, on the other hand, this rationale were applied, and given that the effective concentration of neutral amine substrate depends on the pK_{NH+} of the substrate, the result would be a decrease of several orders of magnitude in the K_M values of the most basic amines ($pK_{NH+} > 7$), while those of the least basic amines ($pK_{NH+} < 7$) would remain unchanged. The overall effect of this data manipulation would be a drastic break in the slope of the Brønsted plot (from $\beta_{nuc} = -0.37$ below $pK_{NH+} = 7$ to $\beta_{nuc} = 0.59$ above $pK_{NH+} = 7$). This practice would not improve upon the observed correlation; the deviation from linearity that would be generated by this treatment of the data argues against its validity.

The validity of the slope of the Brønsted plot was confirmed by repeating the kinetic experiments at pH 6.0 for a smaller set of amine substrates (Table 2). The similarity of the $\log(k_{cat}/K_M)$ values obtained at pH 6.0 to those obtained at pH 7.0 verifies that the resulting shallow negative slope is not simply a reflection of the proportion of free amine according to its relative basicity. Rather, the slope is indicative of the charge developed on the nitrogen at the transition state of the general-base-catalyzed nucleophilic attack of the bound neutral amine on the thiolester acyl enzyme.

Effect of pH on Deacylation Rate. Since an amine must be in its neutral form to be chemically reactive as a nucleophile, there must exist another basic amino acid residue (other than the histidine of the catalytic triad) that is involved in the rapid binding and deprotonation of the predominant primary ammonium substrates, prior to the general-base catalysis of their rate-limiting reaction with the acyl enzyme. The effect of pH on the ionization of this presumed additional base, as well as on the affinity of the enzyme for primary amine substrates, and on the value of K_M due to the state of ionization of the substrate itself, makes pH−rate profiles for aminolysis difficult to interpret in terms of useful mechanistic information (*28*).

Furthermore, as mentioned previously, the kinetic methodology employed herein suffers from certain limitations that make extensive pH−rate studies unfeasible. Nevertheless, over the limited pH range of 6.0−8.0, the hydrolysis reaction and the aminolysis reactions for all of the amine substrates studied were studied and found to be relatively insensitive to pH, displaying broad plateaus on both the pH−V_{max} and pH−K_M profiles (*28*). The absence of significant pH-dependence over this pH range is consistent with a putative general-base whose conjugate acid has an apparent kinetic $pK_a < 6.0$. It is also worthwhile to note that our Brønsted plots constructed both at pH 7.0 and at pH 6.0, located on

Table 2: Kinetic Parameters [k_{cat}, K_M, and $\log(k_{cat}/K_M)$] Obtained According to Equations 2 and 3 for the Reaction of TGase at 25 °C and pH 6.0 (0.1 M MES) with 0.1 mM **5** in the Presence of a Series of Primary Amine Acceptor Substrates

acceptor substrate	k_{cat}[a]	K_M[b]	$\log(k_{cat}/K_M)$[c]
aminoacetonitrile	59.6	0.06	2.997
2,2,2-trifluoroethylamine	43.5	0.58	1.875
glycinamide	42.9	3.21	1.126
2-(ethylthio)ethylamine	55.6	71.0	−0.106

[a] Given in units of min^{-1} and normalized for a specific activity of 14 U/mg; experimental error is estimated at ±10%. [b] Given in units of mM; experimental error is estimated at ±10%. [c] Experimental error is estimated at ±0.05.

Table 3: Kinetic Parameters (k_{cat} and K_M), Obtained According to Equations 2 and 3, and Kinetic Isotope Effects $[(k_{cat}/K_M)^{H_2O}/(k_{cat}/K_M)^{D_2O}]$ for the Reaction of TGase in D_2O, at 25 °C at pD 7.0 (0.1 M MOPS), with 0.1 mM **5** in the Presence of Three Primary Amine Acceptor Substrates

acceptor substrate	k_{cat}[a]	K_M[b]	k_{cat}/K_M	kie[c]
aminoacetonitrile	39.1	0.20	196	5.9
glycine methyl ester	24.7	0.74	33.3	3.4
N-acetyl-L-lysine methyl ester	28.9	3.1	9.32	1.2

[a] Given in units of min^{-1} and normalized for a specific activity of 14 U/mg; experimental error is estimated at ±10%. [b] Given in units of mM; experimental error is estimated at ±10%. [c] Experimental error is estimated at ±20%.

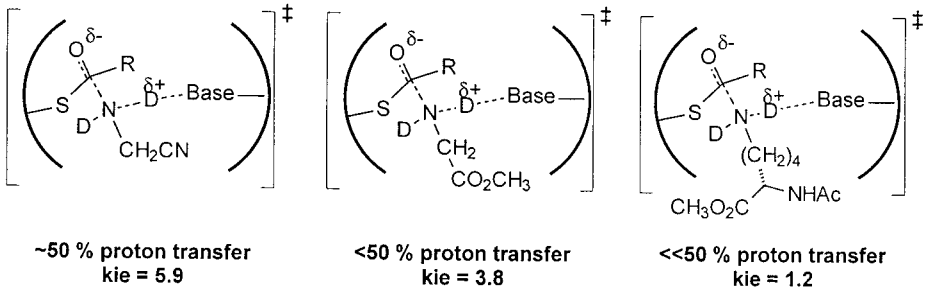


FIGURE 3: Diagrammatic representation of the speculative degree of proton-transfer realized at the transition state of the general-base-catalyzed deacylation reaction in D_2O, according to the relative acidity of the nucleophilic primary amine acceptor substrate. The relative degree of proton transfer correlates with the magnitude of the observed kinetic isotope effects (see Table 3).

this broad plateau, provide information that reflects the mechanism of action of the particular state of ionization of the acyl enzyme at that plateau.

Isotope Effect Studies. If the shallow negative slope of Figure 2 derives from general-base catalysis during deacylation, then the putative proton in flight at the rate-limiting transition state may be expected to give rise to a kinetic isotope effect. To assess this possibility, kinetic parameters were obtained in D_2O for four acceptor substrates: water, aminoacetonitrile, glycine methyl ester, and *N*-acetyl-L-lysine methyl ester. Under the conditions employed, all exchangeable protons would be replaced by deuterons. Furthermore, since pL 7.0 is part of the broad plateau of the pH−rate profiles (*28*), the perturbation of enzyme ionization by the change of solvent would be negligible, leading to the conclusion that the observed differences in reaction rate are kinetic effects and not equilibrium effects. For the reaction with **5** in D_2O, the K_M of **5** was determined to be 0.08 mM and the k_{cat} for hydrolysis was determined to be 16.2 min^{-1}, giving a kinetic isotope effect (in this case, $k_{cat}^{H_2O}/k_{cat}^{D_2O}$) of 1.1. The results of the aminolysis experiments are summarized in Table 3.

In each case, a significant kinetic isotope effect was observed for k_{cat}/K_M, suggesting that there is a proton in flight at the rate-limiting transition state, consistent with general-base catalysis. Furthermore, the magnitude of the observed kinetic isotope effect appears to be proportional to the acidity of the acceptor substrate. Now, the size of a kinetic isotope effect depends on the degree of proton transfer at the transition state, with a maximum effect expected for a transition state that reflects 50% proton transfer (*36*). The degree of proton transfer at the transition state is determined in turn by the acidity of the incoming nucleophile. It then follows that the acidity of the nucleophile could influence the size of the observed isotope effect. The results shown in Table 3 are consistent with this reasoning, in that the largest isotope effects were observed with aminoacetonitrile, the most acidic acceptor substrate studied, and whose transition state should demonstrate the largest degree of proton transfer. Moreover, increasingly less pronounced isotope effects were observed with decreasingly acidic amines, whose transition states would feature relatively lesser degrees of proton transfer. The effect of amine acidity on the degree of proton transfer at the transition state and the corresponding magnitude of the observed kinetic isotope effect is represented schematically in Figure 3.

Given the three-dimensional structure of TGases, it seems reasonable to speculate that the general-base implicated by the kinetic data presented herein is the histidine imidazole of the postulated catalytic triad. By way of amino acid sequence comparison (*19*) with Factor XIIIa, for which the X-ray crystallographic structure is known (*17*), this triad comprises Cys-276, His-334, and Asp-358 in guinea pig liver TGase. It is worthwhile to note that replacement of the catalytic triad histidine of Factor XIIIa with asparagine by site-directed mutagenesis has been shown to abolish enzyme activity (*37*). Experiments of this type with guinea pig liver TGase, as well as further elaboration of the pH−rate dependence of its transamidation mechanism, are currently underway in our laboratory.

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SUPPORTING INFORMATION AVAILABLE

The synthesis of substrate analogue **5** and also tables containing the steady-state reaction rates for the various reactions of guinea pig liver TGase with **5**, in the presence and absence of primary amine acceptor substrates, are available on request (22 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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