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Mechanism of Hydrolysis and Aminolysis of Homocysteine Thiolactone

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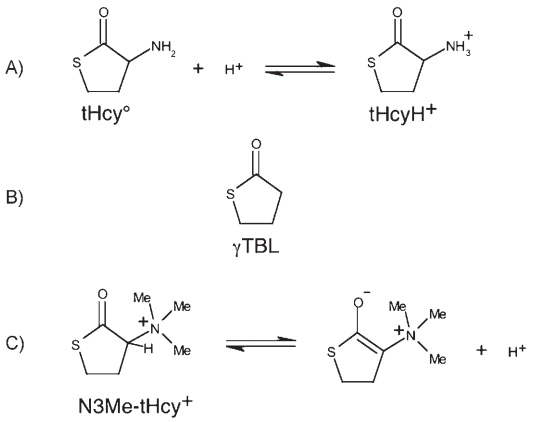
Abstract: Homocysteine thiolactone (tHcy) is deemed a risk factor for cardiovascular diseases and strokes, presumably because it acylates the side chain of protein lysine residues ("N-homocysteinylation"), thereby causing protein damage and autoimmune responses. We analysed the kinetics of hydrolysis and aminolysis of tHcy and two related thiolactones (γ-thiobutyrolactone and *N*-trimethyl-tHcy), and we have thereby described the first detailed mechanism of thiolactone aminolysis. As opposed to the previously studied (thio and oxo)esters and (oxo)-lactones, aminolysis of thiolactones was found to be first order with respect to amine concentration. Anchimeric assistance by the α-amino group of tHcy (through general acid/base catalysis) could not be detected, and the Brønsted plot (nucleophilicity versus pK_a) for aminolysis yielded a slope (β^{nuc}) value of 0.66. These data support a mechanism of aminolysis where the rate-determining step is the formation of a zwitterionic tetrahedral intermediate. The β^{nuc} value and steric factors dictate a regime whereby, at physiological pH values (pH 7.4), maximal reactivity of tHcy is exhibited with primary amine groups with a pK_a value of 7.7; this allows the reactivity of various protein amino groups towards N-homocysteinylation to be predicted.

Keywords: aminolysis · atherosclerosis · homocysteine · hydrolysis · protein modifications

Introduction

Homocysteine (Hcy) is a known risk factor for cardiovascular disease and stroke in humans, although its mechanism of action is largely unknown.[1,2] Recently, the toxicity of Hcy has been attributed, amongst other factors, to homocysteine thiolactone (tHcy, Scheme 1), a product of Hcy editing by tRNA synthetases.[1,2] tHcy was shown to acylate protein lysine side chains ("N-homocysteinylation", Scheme 2) in an irreversible fashion.[2–6]

Due to the presence of a thioester moiety that acts as an electron-withdrawing group, the pK_a value of the amine group of tHcy is 7.1,[7] which is ≈3 units below the pK_a value of ordinary amino groups, for example, the ε-amino group of lysine side chains (pK_a = 10.6). Consequently, N-homocysteinylation causes a modification of the protein's structure and of its isoelectric point. It also attaches a free thiol group at its surface. These changes can induce loss of protein function, denaturation and precipitation and can elicit autoimmune responses.[2,3] N-homocysteinylation has been investigated at the protein level, but surprisingly little attention has so far been devoted to the basic chemical aspects of the reaction.[8] The aminolysis reactions of



Scheme 1. The thiolactones studied: A) Homocysteine thiolactone in its base (tHcy°) and acid forms (tHcyH+), B) γ-thiobutyrolactone (γTBL), and C) *N*-trimethylhomocysteine thiolactone (N3Me-tHcy+) and its enolic tautomer.

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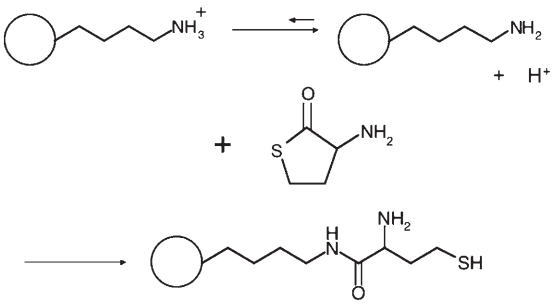
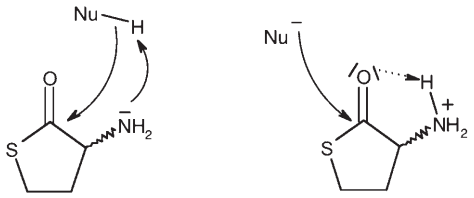


Scheme 2. N-homocysteinylation of protein amino groups.



Scheme 3. Putative models of anchimeric assistance in tHcy: intramolecular general base catalysis (left), and intramolecular general acid catalysis (right). NuH refers to H_2O (hydrolysis) or RNH_3^+ (aminolysis).

esters,[9–14] thioesters[9,11] and oxolactones[15] have been all studied in the past but, to the best of our knowledge, no such study has been performed with thiolactones, let alone with tHcy. In the case of N-homocysteinylation of proteins, several important questions have not been answered yet. What is especially astonishing is that relatively rapid nonenzymatic[3] N-acylation of proteins takes place in the serum at physiological pH values, conditions under which less than one thousandth of the lysine residues are deprotonated, and numerous other nucleophiles (including water) can compete. The steric and electronic factors that govern the reactivity of amino groups with tHcy have not been studied either; in particular, N-terminal amino groups of proteins exhibit lower pK_a values ($\approx$8.5) and are therefore obvious targets for modification, yet they do not seem to preferentially undergo N-homocysteinylation.[2,3] Likewise, it is not known, apart from in the specific case of Lys525 in human serum albumin (HSA),[6] whether and why certain lysine residues react faster than others with tHcy.

To provide further insight into the mechanism of N-homocysteinylation, kinetic studies of hydrolysis and aminolysis of tHcy were performed. Amines with various pK_a values and steric crowding were used to examine the influence of steric and electronic effects on the reaction rates. The pH–rate profiles of hydrolysis and aminolysis of tHcy were constructed, along with the Brønsted plot (amine nucleophilicity versus pK_a value). Our goals were to investigate the mechanisms of both reactions and analyse the steric and electronic factors that govern the reactivity of amino group in proteins. Amongst others, one question that drew our attention regarded the role of the α-amino group of tHcy: Is its influence on the reaction rates merely electrostatic, or does it provide intramolecular catalysis through anchimeric assistance (Scheme 3)? Anchimeric assistance could, for one thing, facilitate the reaction of protonated amino groups, thereby accounting for the rapid N-homocysteinylation of lysine residues at physiological pH values.

To better understand the effects of the α-amino group of tHcy, the hydrolysis and aminolysis of two reference compounds, γ-thiobutyrolactone (γTBL) and *N*-trimethylhomocysteine thiolactone (N3Me-tHcy$^+$; Scheme 1), were also investigated. Comparison of the kinetics of tHcy with those of γTBL allowed us to distinguish between general features of thiolactones and the effect of the α-amino group, for example, anchimeric assistance. The N3Me derivative of tHcy was chosen because it mimics the electrostatic contribution of tHcy's amino group in its protonated state (tHcyH$^+$; Scheme 1), yet it is unable to participate in intramolecular general acid/base catalysis. Analysis of the rates and pH–rate profiles observed with tHcy and the above reference compounds provides no indication for anchimeric assistance by the α-amino group of tHcy. Altogether, the data provide a detailed mechanism for both the hydrolysis and aminolysis of thiolactones in general, and tHcy in particular, and allows the prediction of the reactivity of various protein amino groups towards N-homocysteinylation.

Results

Mechanism of hydrolysis

pH–rate profile of γTBL: The hydrolysis of γTBL (Scheme 1B) was first studied as a simpler model for the hydrolysis of tHcy, and its pH–rate profile was constructed (Figure 1). Equation (1), where $k^{(OH^-/\gamma TBL)}$ and $k^{(H_2O/\gamma TBL)}$ were left as

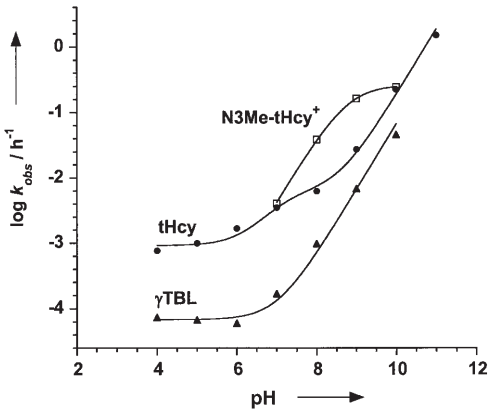


Figure 1. pH–rate profiles of hydrolysis of γTBL, tHcy and N3Me-tHcy$^+$. Logarithmic values of the apparent rate constants of γTBL (▲), tHcy (●) and N3Me-tHcy$^+$ (□) hydrolysis are plotted against pH value. The values reported correspond to the rate constants measured at I = 0.2 M, and 21 ± 1 °C, except for γTBL (pH 4–8) and tHcy (pH 4 and 5) for which the values plotted were extrapolated from measurements at 60 °C. All rates were extrapolated to zero buffer concentration. The estimated maximum of experimental error ranges for the rate constants is ±10% for the rates directly measured and ±25% for the extrapolated rates. Data were fitted to Equation (1) for γTBL, Equation (3) for tHcy and Equation (4) for N3Me-tHcy$^+$, as indicated in the Results section.

floating parameters and $[H_2O]=55\,M$, provided a good fit for the experimental data throughout the pH range.

$$v = (k^{(OH^-/\gamma TBL)}[OH^-]+k^{(H_2O/\gamma TBL)}[H_2O]) \times [\gamma TBL] \quad (1)$$

The pH–rate profile indicates OH^--catalysed hydrolysis for pH > 7 and noncatalysed ("water-catalysed") hydrolysis for pH < 7. The introduction of an H^+ catalysis component into Equation (1) did not provide a better fit to the experimental data and was therefore ignored. The second-order rate constants derived from the fit to Equation (1) are provided in Table 1.

Table 1. Kinetic parameters of hydrolysis of γTBL, tHcy°, tHcyH$^+$ and N3Me-tHcy$^+$.[a]

Rate constants	γTBL	tHcy°	tHcyH$^+$	N3Me-tHcy$^+$
$k^{(OH^-)}$ [$M^{-1}h^{-1}$]	6.5×10^{2}	1.8×10^{3}	5.0×10^{4}	4.2×10^{4}
$k^{(H_2O)}$ [$M^{-1}h^{-1}$]	1.2×10^{-6}	N.D.	1.7×10^{-5}	N.D.

[a] The maximal estimated error range for the second-order rate constants is ±35% (±25% from the experimental error and ±10% from deviations from the fit). N.D. = not determined.

pH–rate profile of tHcy: The general rate law of tHcy hydrolysis is affected by the acidic and basic forms of tHcy (tHcyH$^+$ and tHcy°, respectively), which can exhibit specific acid catalysis, specific base catalysis and water catalysis with different rate constants. It is a sum of six terms,[16] with the concentrations of tHcy° and tHcyH$^+$ being themselves pH dependent [Eq. (2)].

$$\begin{aligned} v = {} & (k^{(H^+/tHcy^o)}[H^+]+k^{(OH^-/tHcy^o)}[OH^-]+k^{(H_2O/tHcy^o)}[H_2O]) \\ & \times[tHcy^o]+(k^{(H^+/tHcyH^+)}[H^+]+k^{(OH^-/tHcyH^+)}[OH^-] \\ & +k^{(H_2O/tHcyH^+)}[H_2O]) \times [tHcyH^+] \end{aligned} \quad (2)$$

Fitting the pH–rate profile to Equation (2) is not sufficient to determine the six second-order rate constants it contains because some of the terms are kinetically indistinguishable (for instance, the term $[OH^-][tHcyH^+]$ has the same variation with pH value as $[H_2O][tHcy^\circ]$). However, additional data indicated below resolve this ambiguity. The pH–rate profile of tHcy (Figure 1) was constructed from pH 4 to pH 11, and a rate law that carries only part of the terms of Equation (2) was sufficient to account for the experimental data [Eq. (3)], with $[tHcyH^+]=[tHcy]_{tot}/(1+10^{(pH-7.1)})$ and $[tHcy^\circ]=[tHcy]_{tot}/(1+10^{(7.1-pH)})$.

$$\begin{aligned} v = {} & k^{(OH^-/tHcy^o)}[OH^-][tHcy^o]+(k^{(OH^-/tHcyH^+)}[OH^-] \\ & +k^{(H_2O/tHcyH^+)}[H_2O]) \times [tHcyH^+] \end{aligned} \quad (3)$$

As for γTBL, acid catalysis was not observed within the pH range surveyed, and tHcy hydrolysis is hydroxide catalysed at pH > 8 and water catalysed at pH < 6. The shoulder appearing for intermediate pH values around the pK_a value of tHcy (7.1) seems to indicate that hydroxide-catalysed hydrolysis is faster for tHcyH$^+$ than for tHcy°. Indeed, pH variations around the pK_a value of tHcy entail variations of tHcyH$^+$ and OH^- concentrations in two opposite ways, thereby giving rise to the observed shoulder, if it is assumed that tHcyH$^+$ is hydrolysed faster that tHcy°. Alternatively, this shoulder could correspond to water hydrolysis of tHcy°. These mechanisms are kinetically indistinguishable and the actual mechanism could be described by either one of these hypotheses or a combination of both.

pH–rate profile of N3Me-tHcy$^+$: To provide further insights about the relative importance of these two descriptions of the pH–rate shoulder and, specifically, to estimate the level of activation by the positively charged α-amino group of tHcyH$^+$, N3Me-tHcy$^+$ (Scheme 1C) was synthesised, and its pH–rate profile was constructed. The replacement of hydrogen atoms by three methyl groups renders impossible the loss of the positive charge on the nitrogen atom and thereby simplifies the rate law and pH–rate profile (Figure 1). The plateau observed at pH > 9 probably corresponds to an acid-obasic equilibrium, that is, to the formation of an enolate (Scheme 1C). The α proton is labile due to the presence of two electron-withdrawing groups (the carbonyl group of the thioester and the positively charged quaternary ammonium). Indeed, in contrast to the results with tHcy, the α proton of N3Me-tHcy$^+$ gives no signal in the proton NMR spectrum in D_2O, a result suggesting a fast exchange with deuterium atoms due to the low pK_a value. The hydrolysis of the zwitterionic enolate form is probably extremely slow, hence the plateau observed. Below pH 8, the rates are linearly proportional to hydroxide concentration, and the experimental data could be readily fitted to Equation (4) to yield $k^{(OH^-/N3Me-tHcy^+)}=4.2\times10^4\,M^{-1}h^{-1}$ and pK_a(N3Me-tHcy$^+$) = 8.8.

$$v = k^{(OH^-/N3Me-tHcy^+)}[OH^-][N3Me\text{-}tHcy^+]_{tot}/(1+10^{(pH-pK_a(N3Me-tHcy^+))}) \quad (4)$$

Mechanism of tHcy hydrolysis: The hydroxide-catalysed hydrolysis occurs 23 times faster with N3Me-tHcy$^+$ than with tHcy° (Table 1). If N3Me-tHcy$^+$ is actually a good model for the protonated form of tHcy (tHcyH$^+$), the latter result is consistent with the facts that tHcyH$^+$ is much more reactive than tHcy° towards hydroxide-catalysed hydrolysis and that hydroxide-catalysed hydrolysis of the former dominates the shoulder part of the pH–rate profile at pH > 7. Indeed, fitting the pH–rate profile of tHcy with Equation (3) gives the following value for the base-catalysed hydrolysis constant of tHcyH$^+$: $k^{(OH^-/tHcyH^+)}=5.0\times10^4\,M^{-1}h^{-1}$. Thus, according to this model, $k^{(OH^-/tHcyH^+)}$ and $k^{(OH^-/N3Me\text{-}tHcy^+)}$ are equal within the margin of experimental error, and the hydroxide-catalysed hydrolysis of tHcyH$^+$ turns out to be 28 times faster than that of tHcy° (Table 1). It seems therefore unlikely that the "water-catalysed" hydrolysis of the neutral form of tHcy significantly participates in the reaction around pH 7–8.

These results are also of significance concerning the propensity of the α-amino group of tHcy to accelerate the reac-

tion rates through anchimeric assistance. First, it appears that intramolecular general acid catalysis by the protonated α-amino group is nonexistent in the case of hydrolysis. There is only a 14-fold difference between $k^{(H_2O/tHcyH^+)}$ and $k^{(H_2O/\gamma TBL)}$ (in comparison to a 28-fold difference between tHcy° and $tHcyH^+$ for the hydroxide-catalysed reaction), which strongly suggests that intramolecular general acid catalysis is not a contributing factor. Second, it is likely that intramolecular general base catalysis provided by the neutral α-amino group is essentially none. Although its exact value cannot be directly determined, a maximum estimate of $k^{(H_2O/tHcy^\circ)}$ can be assessed. Assuming that water catalysis of tHcy° accounts fully for the presence of the shoulder in the pH–rate profile of tHcy (which is the most unlikely hypothesis), we find $k_{max}^{(H_2O/tHcy^\circ)} \approx 1.1\times10^{-4}\,M^{-1}h^{-1} \approx 7\times k^{(H_2O/tHcyH^+)}$. Thus, $k^{(H_2O/tHcy^\circ)}$ is of the same order of magnitude as $k^{(H_2O/tHcyH^+)}$, species for which intramolecular general base catalysis cannot occur. Consequently, proficient anchimeric assistance through the neutral amino group of tHcy is very unlikely to occur in the case of hydrolysis.

To summarise, the hydroxide-catalysed hydrolysis occurs with $tHcyH^+$ approximately 28 times faster than with tHcy° and approximately 77 times faster than with γTBL. The water-catalysed hydrolysis occurs with $tHcyH^+$ approximately 14 times faster than with γTBL. Electrostatic effects largely account for these differences, and no contribution of the α-amino group of tHcy through intramolecular general acid/base catalysis was identified.

Mechanism of tHcy aminolysis

Steric effects on aminolysis of tHcy: The rates of aminolysis of tHcy by glycinamide, L-leucinamide and tris(hydroxymethyl)aminomethane were measured at pH 8 with amine concentrations ranging from 0.025–0.4 M. These amines have similar pK_a values but different steric crowding on the α-carbon atom. The apparent rates measured were plotted against the amine concentration and are presented in Figure 2. The data could be fitted to a straight line with a good correlation, which indicates that, within the range of concentrations surveyed, the rate of aminolysis of tHcy shows a first-order dependency with respect to the total concentration of amine.

As expected, the rate of aminolysis decreases when steric crowding of the α-carbon atom increases. Despite almost identical pK_a values, glycinamide reacts approximately 9 times faster than L-leucinamide and at least 22 times faster than tris(hydroxymethyl)aminomethane. However, since all three amines absorb at around 200 nm, the actual formation of the amide bond could not be detected, and tris(hydroxymethyl)aminomethane, in particular, could also accelerate the disappearance of tHcy through general base-catalysed hydrolysis. Thus, the above values represent a minimum estimate for the steric effects.

Electronic effects on aminolysis of tHcy: The pH–rate profiles of tHcy aminolysis with aminocaproic acid (ACA), 2-methoxyethylamine (MEA), propargylamine (PAA), glycinamide (GA) and 2,2,2-trifluoroethylamine (FEA) were constructed. These amines possess varying pK_a values and nonhindered α carbon atoms. Figure 3 presents the logarithmic values of the apparent rate constants for a total amine concentration of 25 mM, plotted against pH value. The experimental data could be readily fitted to Equation (5), where $k^{(RNH_2/tHcyH^+)}$ and $k^{(RNH_2/tHcy^\circ)}$ were left as floating pa-

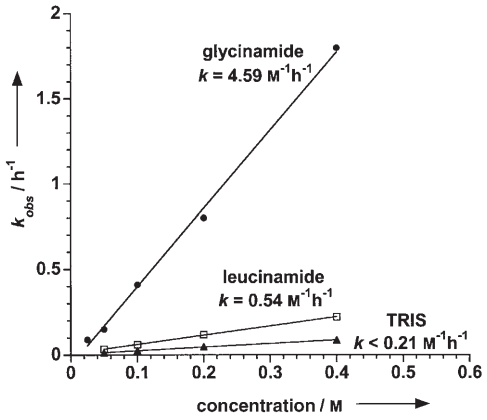


Figure 2. Rates of aminolysis of tHcy by glycinamide, L-leucinamide and tris(hydroxymethyl)aminomethane (21±1°C, I=0.2 M). The apparent first-order rate constants of disappearance of tHcy in aqueous solutions (pH 8) of glycinamide (pK_a=8.0), L-leucinamide (pK_a=7.8) and tris(hydroxymethyl)aminomethane (pK_a=8.0; for pK_a values, see references [17–19]) were plotted against amine concentration. The rates of tHcy hydrolysis under the same conditions were substracted, and the data were fitted to a straight line whose slope and intercept were left as floating parameters. The slopes correspond to the second-order rate constant of aminolysis at pH 8 (in $M^{-1}h^{-1}$). TRIS= tris(hydroxymethyl)aminomethane.

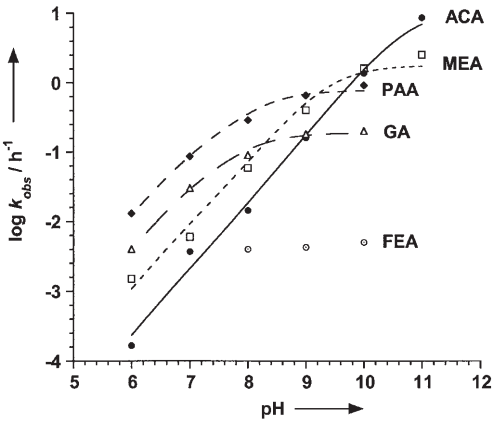


Figure 3. pH–rate profiles of aminolysis of tHcy (21°C, I=0.2 M, [Amine]=25 mM). Logarithmic values of the apparent rate constants of aminolysis of tHcy by aminocaproic acid (ACA, ●), 2-methoxyethylamine (MEA, □), propargylamine (PAA, ◆), glycinamide (GA, △) and 2,2,2,-trifluoroethylamine (FEA, ○) were plotted against pH value. The rates were extrapolated to zero buffer concentration, and the rates of hydrolysis at the same pH value were substracted. The maximum estimated error range for the apparent rate constants is ±20%. Data were fitted to Equation (5) as indicated in the Results section.

rameters and RNH_2 refers to the neutral (basic) form of the attacking amine ($[RNH_2]=[Amine]_{tot}/(1+10^{(pK_a-pH)})$); the pK_a values of the various amines and the second-order rate constants determined by this fit are indicated in Table 2.

$$v = (k^{(RNH_2/tHcyH^+)}[tHcyH^+]+k^{(RNH_2/tHcy^o)}[tHcy^o]) \times [RNH_2] \quad (5)$$

Table 2. Kinetic parameters of aminolysis of tHcy (21 °C, $I=0.2$ M).[a]

Rate constants	ACA	MEA	PAA	GA	FEA
$k^{(tHcy^\bullet)}$ [$M^{-1}h^{-1}$]	440	70	30	7	0.2
$k^{(tHcyH^+)}$ [$M^{-1}h^{-1}$]	600	110	80	16	N.D.
$k^{(tHcyH^+)}/k^{(tHcy^\bullet)}$	1.4	1.6	2.7	2.2	N.D.
literature pK_a values[17–19]	10.8	9.4	8.2	8.0	5.6

[a] The maximal estimated error range for the second-order rate constants is ±30% (±20% from the experimental error and ±10% from deviations from the fit). N.D. = not determined.

From the pH–rate profiles, we can deduce several important conclusions concerning the mechanism of aminolysis of tHcy. First, the rates of aminolysis of tHcy are directly proportional to the concentration of the neutral (basic) form of the attacking amine and obey a first-order regime. The effect of the charged (acidic) form of the attacking amine on the rate of aminolysis of tHcy is essentially none, which rules out its participation in the reaction. Besides the fact that the experimental data can be fitted to an equation in which the charged form of the attacking amine (RNH_3^+) is not taken into account, it is immediately visible that the rates correlate with the concentration of the basic form of the amine: below the pK_a value of each amine, a straight line with a slope of +1 is observed, whereas a plateau for $\log k_{obs}$ appears above the pK_a value (Figure 3). As it is the case for hydrolysis, the α-amino group of tHcy does not seem to participate in the mechanism through anchimeric assistance.

Second, the aminolysis of tHcy is not hydroxide catalysed. Several instances report that the aminolysis of esters or thioesters is base catalysed,[10–14] in particular, by the attacking amine itself (which requires the introduction of quadratic terms in the rate law) or by hydroxide ions. In the present case, no catalysis by hydroxide ions seems to occur, since the apparent rates measured are pH independent above the pK_a value of the attacking amine. In order to determine whether this was particular to homocysteine thiolactone or a general feature of thiolactones, the pH–rate profiles of aminolysis of γTBL with aminocaproic acid, 2-methoxyethylamine and glycinamide was constructed; the same behaviour was observed (data not shown). Additionally, the average ratio for $k^{(RNH_2/tHcy^\bullet)}/k^{(RNH_2/\gamma TBL)}$ is approximately 9±1, which is similar to the value determined for the hydroxide-catalysed hydrolysis ($k^{(OH^-/tHcy^\bullet)}/k^{(OH^-/\gamma TBL)} \approx 3$; Table 1).

Third, unlike the rate of hydrolysis, the rate of aminolysis is largely independent of the protonation state of tHcy. Unlike the pH–rate profile of hydrolysis (Figure 1), the pH–rate profile of aminolysis does not present a shoulder around the pK_a value of tHcy (7.1). This reveals that the rates of aminolysis of tHcy° and $tHcyH^+$ are of the same order of magnitude, which is also apparent from the rate constants obtained from the fit of the pH–rate profile to Equation (5) (Table 2).

We have also tried to measure the rate of aminolysis of N3Me-$tHcy^+$, but we could not detect any aminolysis at pH 8 in the presence of 0.1 M of aminocaproic acid, 2-methoxyethylamine or glycinamide, nor in the presence of 0.1 M of ammonium chloride (data not shown), with the latter showing that steric effects do not account for the low reactivity of N3Me-$tHcy^+$ towards amines. Assuming an experimental error of 20% on the measured second-order rate constants, we assess that $k^{(RNH_2/N3Me\text{-}tHcy^+)} \leq k^{(RNH_2/tHcy^\bullet)}/40$, but at this stage we cannot provide an unambiguous explanation for this difference.

Brønsted plot: The logarithmic values of $k^{(RNH_2/tHcy^\bullet)}$ were plotted against the pK_a value of the attacking amine (a Brønsted plot, Figure 4). Apart from a slight discrepancy observed for propargylamine, the data could be fitted with good correlation to a straight line with a slope of $\beta^{nuc}(tHcy^\bullet)=0.66$. (Propargylamine exhibits an aminolysis rate that is approximately fivefold higher than expected for its pK_a value, possibly owing to an α effect by the adjacent triple bond.) This suggests that the mechanism and the rate-determining step of tHcy aminolysis remain constant throughout the range of pK_a values surveyed.[14,15] The logarithmic values of $k^{(RNH_2/tHcyH^+)}$ are also represented, although the range of pK_a values is restricted. (Due to technical reasons, the second-order rate constant of $tHcyH^+$ aminolysis with 2,2,2-trifluoroethylamine could not be measured.) With the exception of propargylamine, a linear correlation with pK_a value is observed, thereby providing an estimated slope of $\beta^{nuc}(tHcyH^+) \approx 0.56$.

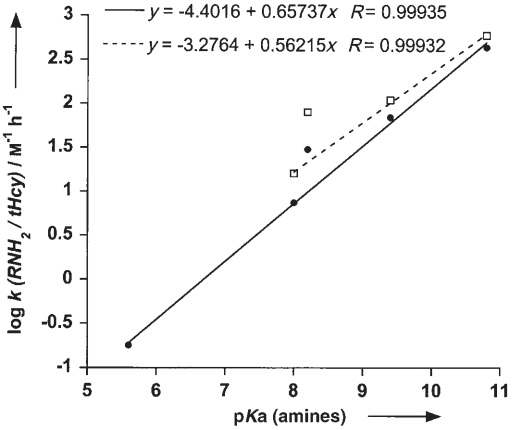
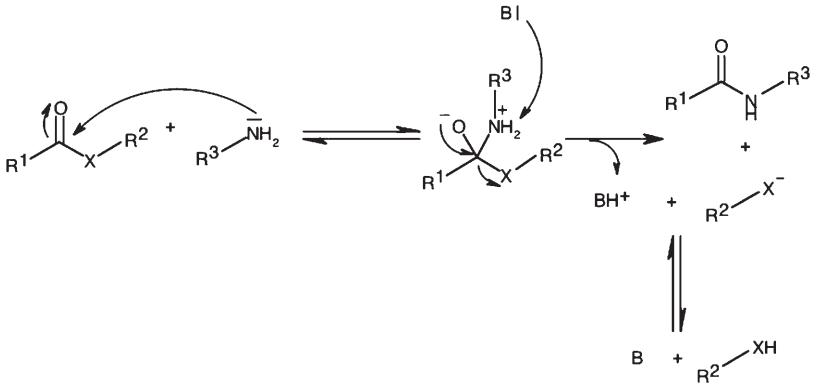


Figure 4. Brønsted plot for tHcy aminolysis. Logarithmic values of the second-order rate constants of tHcy° (solid line) and $tHcyH^+$ (dashed line) aminolysis with various amines were plotted against the pK_a value of the attacking amine. The data, excluding propargylamine, were fitted to straight lines to give slopes (β^{nuc}) of 0.66 (tHcy°) and 0.56 ($tHcyH^+$).

Discussion

The mechanism of tHcy hydrolysis and aminolysis: This study indicates that the α-amino group activates tHcy (relative to γTBL) towards both hydrolysis (by ≈3-fold for tHcy° and ≈77-fold for $tHcyH^+$ for hydroxide-catalysed hydrolysis; Table 1) and aminolysis (by ≈9-fold for tHcy° and <20-fold for $tHcyH^+$; Table 2). This activation seems to result from through-bond electrostatic effects, since neither the hydrolysis nor the aminolysis of tHcy involves significant general base or acid catalysis through the α-amino group. This is in accordance with the intuitive representation of the geometry of the reactants in the corresponding transition states: intramolecular general base catalysis would force the nucleophile to attack on the bulky side of tHcy, while intramolecular general acid catalysis would lead to the formation of a loose five-membered hydrogen-bonded ring. Both of these models seem to be irrelevant to tHcy hydrolysis and aminolysis. Indeed, it has been pointed out that intramolecular general acid/base catalysis demands a very precise positioning of the groups involved[16,20–23] and would otherwise only have minor effects on the reaction rates.[22]



Scheme 4. A simplified scheme of the mechanism of aminolysis of esters (X=O) and thioesters (X=S). For oxoesters, the second step (breaking of the C–X bond) was shown to be rate determining, whereas for thioesters the first step (formation of the tetrahedral intermediate) is rate determining.[9,11] B=base.

Our results also highlight the fact that the basic and acidic forms of tHcy exhibit different reactivities towards different nucleophiles. Both $tHcyH^+$ and its methylated counterpart ($N3Me\text{-}tHcy^+$) were shown to react with hydroxide ions approximately 25 times faster than tHcy°. By contrast, the rate constants of aminolysis of $tHcyH^+$ and of tHcy° are of the same order of magnitude (Table 2), and $N3Me\text{-}tHcy^+$ exhibits unexpectedly low reactivity towards amines. The noncatalysed (water) hydrolysis seems to follows the same pattern as that with amines. Although the rates of tHcy° hydrolysis by water could not be directly determined, we measured $k^{(H_2O/tHcyH^+)}/k^{(H_2O/\gamma TBL)} \approx 14$, whereas $k^{(OH^-/tHcyH^+)}/k^{(OH^-/\gamma TBL)} \approx 77$. Thus, the difference of reactivity between $tHcyH^+$ and tHcy° (as well as γTBL) is much more pronounced for the hydroxide-catalysed hydrolysis than for the water-catalysed hydrolysis and for aminolysis. The reactivity of the nucleophile therefore correlates with its charge: neutral nucleophiles (water, amines) react with both forms of tHcy with similar rates, whereas anionic nucleophiles (hydroxide) react much faster with the protonated form of tHcy.

General notes on the mechanism of thiolactone hydrolysis and aminolysis: Our results allow the description of a general reaction mechanism of thiolactones that has not been available thus far. However, the aminolysis reactions of (oxo)esters,[9–14] thioesters[9,11] and (oxo)lactones[15] have been thoroughly studied, both experimentally and computationally, and several important features have been reported. First, relative to the simple mechanisms with anionic nucleophiles, aminolysis is complicated by the involvement of several proton-transfer steps.[9] Hence, a more complex mechanism (for example, that in Scheme 4) and rate laws with linear as well as quadratic terms in amine and hydroxide concentration become necessary.[10–12,15] Proton transfer can occur in an intra- as well as intermolecular fashion, hence the role of base catalysis (by a hydroxide, or a second amine molecule) observed in the aminolysis of esters, thioesters and lactones.[10–15] Second, whereas oxoesters and thioesters exhibit similar reactivities towards hydrolysis, aminolysis occurs more readily with thioesters than with oxoesters. This has been rationalised in terms of a change in the rate-determining step[9,11] (opening of the C–O bond for oxoesters, nucleophilic attack for thioesters, since thiolate is a better leaving group than alkoxide) and by the higher electrophilicity of the thioester bond due to the smaller degree of resonance of the sulphur lone electron pair.[9] Third, the aminolysis and hydrolysis[24] of lactones are much faster than those of the corresponding open-chain esters. Indeed, the opening of the C–O bond is facilitated in lactones by the chain randomisation and the relief of steric strains that it induces.[15]

It follows from these studies that both thioesters and oxolactones react faster than the corresponding oxoesters with amines and that, in both cases, the transition state is shifted upstream (that is, the opening of the C–O or C–S bond is easier than for oxoesters). We may therefore assume that the rate-determining step of tHcy aminolysis is the nucleophilic attack of the amine, that is, once a tetrahedral intermediate is formed, the proton transfer and the breakage of the C–S bond will occur very rapidly. Our data support this hypothesis. First, no quadratic term in amine or hydroxide catalysis is detected in the rate law (that is, aminolysis is not base catalysed), and proton transfer thus does not occur in

the rate-determining step. Second, the fact that amines, but not hydroxide ions, react with similar rates with tHcy° and $tHcyH^+$ is consistent with both reactions exhibiting "early", possibly zwitterionic, tetrahedral transition states that follow the nucleophilic attack by an amine or OH^- ion. If this assumption is followed, although the protonation of the α-amino group of tHcy would destabilise the ground state in both cases, it may stabilise the transition state for hydrolysis but destabilise that of aminolysis, hence the large differences in reactivity observed between $tHcyH^+$ and tHcy° in the case of hydrolysis but not aminolysis. This might be the reason for the very low rate of aminolysis of N3Me-$tHcy^+$, where the presence of a permanent positive charge on the α-amine dramatically decreases the rate (by at least 40-fold relative to tHcy°). Third, the linear fit of the Brønsted plot indicates that the rate-determining step of tHcy aminolysis remains constant throughout the pK_a range surveyed,[14,15] which seems to refute the possibility of a "late" transition state. Indeed, it is highly improbable that the nucleophilic attack would not be rate determining for low pK_a value amines of poor nucleophilicity, such as 2,2,2-trifluoroethylamine. The comparatively high β^{nuc} value (Figure 4) suggests that a partial positive charge is located on the nucleophilic nitrogen atom in the transition state and could indicate the presence of a zwitterionic tetrahedral intermediate (Scheme 4).

To summarise the mechanistic discussion, this study points out significant differences between the mechanism of thiolactone aminolysis versus that of lactones, esters and thioesters. General base catalysis, by either an additional amine molecule or a hydroxide ion is not required. The rate-determining step is the nucleophilic attack by the amine and not the opening of the C–O or C–S bond. Finally, tHcy aminolysis and hydrolysis do not involve efficient anchimeric assistance from the α-amino group of tHcy, and the propensity of tHcy to react with amines stems primarily from the high reactivity of thiolactones and the electron-withdrawing properties of the α-amino group in both its neutral and protonated states.

Implications for N-homocysteinylation of proteins: The mechanistic insights also enable the prediction of the propensity of protein lysine side chains to undergo N-homocysteinylation in vivo. Let us assume first that protein lysine residues exhibit the same reactivity as free lysine in solution ($pK_a = 10.6$). We can thus evaluate the rates of tHcy hydrolysis and aminolysis at the average serum pH value (7.4) to be $k^{(hydrolysis)} \approx 4.8 \times 10^{-3}\ h^{-1}$ (first-order apparent rate constant) and $k^{(aminolysis)} \approx 4.1 \times 10^{2}\ M^{-1}\ h^{-1}$ (second-order rate constant). The aminolysis rate would therefore match the hydrolysis rate at a total lysine concentration of approximately 19 mM (corresponding to approximately 1.2×10^{-5} M deprotonated lysine). Indeed, this concentration (19 mM) is comparable to the concentration of lysine side chains in the serum. The total concentration of HSA lysines, for instance, is approximately 40 mM.[25] Since HSA is the most abundant protein in serum, and by far the major target of homocysteinylation,[6] our results suggest that in vivo protein N-homocysteinylation may effectively compete with tHcy hydrolysis.

However, in contrast to this simplistic model, the pK_a value of the lysine side chains of proteins may significantly deviate from the solution value of 10.6.[26] A decrease of several units in the pK_a value is not uncommon for protein side chains: an unusually reactive lysine residue with a pK_a value of 5.9 has been identified in acetoacetate decarboxylase,[27] and a pK_a value of 7.9 was reported for Lys199 in HSA.[25] Generally speaking, pK_a depression of a lysine can occur if it is buried in a hydrophobic environment[28,29] or if it neighbours positively charged amino acids (such as histidine, arginine or another lysine[29]). What then is the optimal pK_a value for tHcy aminolysis? If it is assumed that protein lysine residues would react with the same rates as nonhindered primary amines with the same pK_a value in solution, the dependency of the relative rate of aminolysis on the pK_a value of the attacking amine at the physiological pH value (7.4) is given by Equation (6).

$$\nu(pK_a) \propto 10^{(\beta^{nuc}(tHcy^{o}) \times pK_a(amine))} / (1 + 10^{(pK_a(amine) - 7.4)}) \qquad (6)$$

For $\beta^{nuc} = 0.66$ (Figure 3), this function admits a maximum at a pK_a value of 7.7, thereby indicating that an amine with a pK_a value of 7.7 should react with tHcy approximately 6.5 times faster than free lysines in solutions.[30]

This model could therefore explain why certain lysine side chains are preferential sites for N-homocysteinylation. For instance, the high reactivity of Lys525 in HSA towards N-homocysteinylation was attributed to a cross-talk with Cys34.[6] Alternatively, this reactivity could be accounted for by a lower pK_a value. In fact, Lys525 is one of the two most reactive lysines in HSA and it proficiently reacts with other kinds of nucleophiles that do not contain a thiol group (for example, it is, in particular, a predominant site for nonenzymatic glycosylation).[25]

In general, the existence of preferential sites for N-homocysteinylation seems a priori paradoxical, given that the rates of homocysteinylation measured for various proteins correlate with their content of lysine and with the rate of aminolysis measured for free lysine in solution.[3] However, as demonstrated previously,[27] significant variations of pK_a value concern only a small fraction of protein lysine residues. Consequently, if bulk rate constants are measured, the high reactivity of a few activated lysines is largely masked by the vast majority that possess a normal pK_a value.[3]

Our model may also explain why the N termini of proteins that exhibit low pK_a values (≈ 8.5) do not comprise a primary target for N-homocysteinylation.[2] At pH 7.4, the lower pK_a value accounts for a mere 5-fold increase in the rate of N-homocysteinylation compared to free lysine, whereas steric effects (unlike lysine side chains, the N termini possess a secondary carbon substituent) may induce up to 10-fold lower rates (Figure 2). These two effects roughly cancel each other out, and it therefore seems that protein N termini have no preference over lysine side chains. A statistical argument could then explain why homocysteinylation

of N-termini could not be detected: since there is only one N-terminal amino acid and many lysines per protein, the propensity of N-termini to undergo N-homocysteinylation remains undetectable in bulk measurements.

Experimental Section

Kinetics: Chemicals were purchased from Aldrich Chemicals Co. and Acros Chemicals. Spectrophotometric measurements were performed on a microtiter plate reader (PowerWave HT Microplate Scanning Spectrophotometer; optical length ≈ 0.5 cm).

Hydrolysis: The hydrolysis of tHcy, γTBL and N3Me-tHcy$^+$ was followed spectrophotometrically by monitoring the decrease in the absorbance of the thioester bond at $\lambda = 240$ nm[2] ($\varepsilon = 2300 \pm 100$ OD $\times$ M^{-1}). tHcy hydrolysis was found to go to completion in all of the conditions surveyed, and infinity points were stable. The values of k_{obs}, the pseudo-first-order rate constants, were computer calculated by using a rigorous least squares procedure; they were found to be independent of the initial concentration of tHcy (0.5 mM in most cases). Various buffers were used: acetate (pH 4–6), phosphate (pH 6–8), borate (pH 8–10) and carbonate (pH 11). Mild buffer catalysis was observed with phosphate and acetate under the conditions of the experiments (50 mM buffer). When necessary, the rates were extrapolated to zero buffer concentration. Ionic strength was kept constant at 0.2 M by supplementation with NaCl. Most of the apparent rate constants were measured directly at room temperatures (21 °C ± 1 °C). In case the rates at 21 °C were too slow to provide reliable data (pH 4 and 5 for tHcy, pH 4–8 for γTBL), the experiments were carried out at 60 °C: the apparent rates at 60 °C were measured from pH 4–10 for tHcy and γTBL. The ratio k(60 °C)/k(21 °C) was found to be constant within the margin of experimental error and equals 40 ± 6. The apparent rate constants at 21 °C were extrapolated by using this ratio, while assuming that it is pH independent. When the measurements were duplicated (tHcy, pH 6, 7 and 9; N3Me-tHcy$^+$, pH 8) or triplicated (tHcy, pH 8 and 10), the standard deviation observed was less than ±10 %. The experimental error was thus assessed to 25 % (10 % (standard deviation)+15 % (temperature factor)).

Aminolysis: tHcy aminolysis reactions were monitored by following the absorbance of the solution of tHcy at 240 nm, which yielded a first-order apparent rate constant after substraction of the background of hydrolysis. Amide bond formation was also monitored directly by following the absorbance increase at 204 nm. (This wavelength was determined with 2-amino-4-mercapto-*N*-propylbutanamide synthesised from tHcy and propylamine.) The initial concentration of tHcy was 0.5 mM. In order to obtain pseudo-first-order rate constants, the attacking amine was used in excess. In most cases, the amine concentration was fixed at 25 mM; however, in cases when 25 mM amine were not sufficient to overcome the background hydrolysis, higher concentrations of amines (up to 200 mM) were used. To construct the pH–rate profile, the data were extrapolated to a concentration of 25 mM amine, with the assumption that the aminolysis of tHcy was first order in amine concentration. This assumption was verified by the experiments carried out with varying concentrations of glycinamide, L-leucinamide and tris(hydroxymethyl)aminomethane. Aminolysis was studied under the same buffer and ionic-strength conditions as hydrolysis, except that all reactions were carried out at 21 °C ± 1 °C. The dependency of the rates upon buffer concentration was investigated for glycinamide from pH 6–10 by variation of the buffer concentration and extrapolation to zero buffer concentration: the influence of buffer concentration was found to be feeble, and aminolysis rates were corrected accordingly. The experiments performed at pH 8 with varying concentrations of glycinamide, L-leucinamide and tris(hydroxymethyl)aminomethane were carried out without buffer, since the attacking amine itself can act as a buffer in the vicinity of its pK_a value. The aminolysis experiments of tHcy with aminocaproic acid and 2-methoxyethymlamine at pH 9 and 10 were duplicated, and the standard deviation observed was less than ±20 %. Aminolysis of γTBL and N3Me-tHcy$^+$ was investigated by following the same protocol as for tHcy.

Synthesis:

N3Me-tHcy$^+$: Homocysteine thiolactone hydrochloride (500 mg, 3.26 mmol), methyl iodide (1215 μL, 19.6 mmol, 6 equiv) and triethylamine (1810 μL, 52.1 mmol, 16 equiv) were dissolved in acetonitrile (≈10 mL) and stirred at room temperature. The reaction was monitored by thin-layer chromatography (eluent: 5 % methanol in dichloromethane). A white precipitate appeared after approximately 15 min. After 1.5 h, the reaction mixture was cooled to 4 °C for 15 min, and the precipitated product (78 mg; yield = 8 %, purity ≥ 95 %) was collected; ^{1}H NMR (250 MHz, D_2O, tetramethylsilane (TMS)): $\delta = 2.52$ (m, 1H), 2.80 (m, 1H), 3.16 (s, 9H), 3.31 ppm (m, 1H).

*2-Amino-4-mercapto-*N*-propylbutanamide*: Homocysteine thiolactone hydrochloride (200 mg, 1.30 mmol) and propylamine (321 μL, 4.90 mmol, 3 equiv) were dissolved in dichloromethane (20 mL) and stirred at room temperature. The reaction was followed by thin-layer chromatography (eluent: dichloromethane). After 24 h, the reaction mixture was cooled to 4 °C for 2 h and filtered under vacuum. The filtrate was evaporated and dried under vacuum, and an oily product (194 mg; yield = 68 %, purity ≥ 80 %) was collected; ^{1}H NMR (250 MHz, $CDCl_3$, TMS): $\delta = 0.76$ (t, 3H), 1.39 (m, 2H), 1.80 (m, 2H), 2.43 (t, 1H), 3.04 (m, 3H), 3.49 ppm (t, 2H).

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[30] Equation (6) is rigorously correct only if $\beta^{nuc}(tHcy^{\bullet}) = \beta^{nuc}(tHcyH^{+})$. Since $\beta^{nuc}(tHcyH^{+})$ could not be measured with accuracy, its contribution was not taken into account. This hardly affects the results exposed thereafter because $\beta^{nuc}(tHcy^{\bullet})$ and $\beta^{nuc}(tHcyH^{+})$ differ only slightly.

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