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Mechanism of the Condensation of Homocysteine Thiolactone with Aldehydes

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Abstract: Chemical reactivity of homocysteine thiolactone (HTL) has been implicated in cardiovascular disease. Owing to its aminoacyl-thioester character, HTL undergoes facile electrophilic and nucleophilic reactions at its amino and activated-carboxyl group, respectively. To gain insight into the mechanism of the reactions involving its amino group, the kinetics of the condensation of homocysteine thiolactone with formaldehyde, acetaldehyde, and pyridoxal phosphate, were analyzed in the pH range from 5 to 10. The reactions were first order with respect to HTL, aldehyde, and hydroxide ion concentrations. Of the two ionic species of HTL ($pK_a = 6.67 \pm 0.05$), the acid form HTL^+ was ~100-fold more reactive than the base form HTL^0. The reactions of HTL with aldehydes involve intermediate adducts. The conversion of the intermediate carbinolamine to a product, 1,3-tetrahydrothiazine-4-carboxylic acid or its 2-substituted analogue, occurs in a two-step reaction. The first step involves hydrolysis of the thioester bond in the intermediate, facilitated by anchimeric assistance by the oxygen of the carbinolamine group of the intermediate. The second step involves an attack of the liberated thiolate on the aldehyde-derived carbon of the intermediate, affording 1,3-tetrahydrothiazine-4-carboxylic acid or its 2-substituted analogue. An unusual feature of these reactions is that the formation of the carbinolamine group increases the reactivity of the thioester bond of HTL ~10^4-fold. The facile formation of tetrahydrothiazines may contribute to HTL elimination from the human body.

Keywords: aldehydes · atherosclerosis · homocysteine thiolactone · reaction mechanisms · tetrahydrothiazine

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is found as a normal metabolite in all three domains of life. Although Hcy is a normal metabolite, its excess can be extremely toxic to human,[1–3] animal,[4] yeast,[5–7] and bacterial cells.[8] In humans, excess Hcy is linked to cardiovascular[9,10] and neurodegenerative diseases, such as Alzheimer's.[11] The strongest evidence that Hcy plays a causal role in cardiovascular disease comes from studies of hyperhomocysteinemia in animal models.[9] A recent study shows that high risk stroke patients do benefit from lowering of plasma Hcy by vitamin supplementation.[12]

Why Hcy is toxic is not entirely clear and is a subject of intense studies.[9,13] One hypothesis suggests that the aminoacyl-thioester homocysteine thiolactone,[6,14,15] a product of Hcy editing during protein synthesis, contributes to Hcy toxicity and is linked to atherosclerosis in humans.[13,16–18] The formation of HTL can be detrimental for two reasons. First, it requires ATP and thus causes nonproductive consumption of cellular energy.[5,6] Second, HTL is a reactive intermediate that causes protein N-homocysteinylation through the formation of isopeptide bonds with ε-amino groups of protein lysine residues.[13,16–23] Resulting protein damage necessitates removal of N-Hcy-proteins by proteolytic degradation, which would further deplete cellular energy and limit cell growth. HTL appears to be more toxic to human cells than Hcy.[3] N-Hcy-protein is also toxic and induces an autoimmune response, which is associated with cardiovascular disease and stroke in humans.[24,25] The mechanism of N-homocysteinylation of protein lysine side chains has been studied in the context of human atherosclerosis.[13,16–23]

Because of its dual aminoacyl-thioester character, HTL undergoes not only nucleophilic, but also electrophilic reactions.[13] Mechanisms of reactions of amines or amino acids

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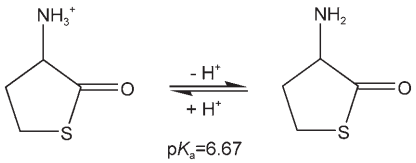
with aldehydes have been studied in the past.[26–29] However, no mechanistic studies of analogous electrophilic reactions involving HTL have been reported. Because of its involvement in human cardiovascular disease, it is important to understand the fundamental chemistry of Hcy and its derivatives.

To gain insight into the mechanism of the reactions involving its amino group, the kinetics of reactions of HTL with naturally occurring aldehydes, such as formaldehyde, acetaldehyde, and pyridoxal phosphate were examined. In particular, the role of acid and base forms of HTL (Scheme 1) was studied. An unusual feature of these reactions is that the formation of a carbinolamine adduct of HTL increases the reactivity of the thioester bond of HTL ~10^4-fold.



Scheme 1. Acid and base forms of HTL.

Results and Discussion

pK_a value of HTL: During the course of these studies it became necessary to determine the pK_a value of HTL. The literature value[30] of 7.1 did not fit our data and appeared to be incorrect. The pK_a value of HTL, measured by potentiometric titration was found to be 6.67 ± 0.05 from eight independent runs on three separate occasions. The reasons for the discrepancy are unclear because the authors[30] give no information regarding how the pK_a of HTL was determined. The pK_a value of 6.67 ± 0.05 for the amino group of HTL, exceptionally low compared to pK_a values of 8.0 for aminoacyl esters,[31] is accounted for by the electron-withdrawing effects of the sulfur.

Tetrahydrothiazine is a stable, readily formed HTL adduct: It is known that the reaction of HTL with formaldehyde affords stable 1,3-tetrahydrothiazine-4-carboxylic acid.[32,33] Identical tetrahydrothiazine is also formed in the reaction of Hcy with formaldehyde.[32,33]

However, basic chemical aspects of the tetrahydrothiazine formation reactions have not been studied. In particular, tetrahydrothiazine formation from HTL requires HTL ring opening, but how this happens was not clear.

Condensation of HTL with aldehydes involves an intermediate: HTL, in contrast to Hcy, exhibits a unique thioester absorption spectrum in UV with a maximum at 240 nm.[13] The characteristic UV absorption at 240 nm disappears during reaction of HTL with aldehydes, which offers an opportunity to examine the mechanism of the conversion of HTL to tetrahydrothiazines.

The rate of condensation of HTL with a large molar excess of formaldehyde or acetaldehyde, measured spectrophotometrically at 240 nm, was first order with respect to HTL. At low aldehyde concentrations, the pseudo-first-order constants were found to increase linearly with the aldehyde concentration, but at high concentrations the rates level off and become less dependent on aldehyde concentrations (Figure 1a). The nonlinear kinetics suggest the formation of an intermediate. Eaddie–Hofstee plots (k_{obs} versus k_{obs} [aldehyde]$^{-1}$) of the data (illustrated for the reaction of HTL with acetaldehyde in Figure 1b) give the maximum pseudo-first-order rate constant at high aldehyde concentrations (that is the rate constant for the conversion of the intermediate to product) as the intercept at the ordinate, and the slope gives a negative value of the reciprocal of the equilibrium constant for the formation of the intermediate. The equilibrium and rate constants for the reactions of formaldehyde and acetaldehyde with the cationic form HTL^+ (at pH 5.34–5.79) and the neutral form HTL^0 (at pH 7.40–8.01) are listed in Table 1. The equilibrium constants for adduct formation between HTL^+ or HTL^0 and formaldehyde or acetaldehyde (Table 1) are similar to those determined for adduct formation between acid ($K = 3.2\,M^{-1}$) or base ($K = 15.0\,M^{-1}$) forms of imidazole and formaldehyde.[27]

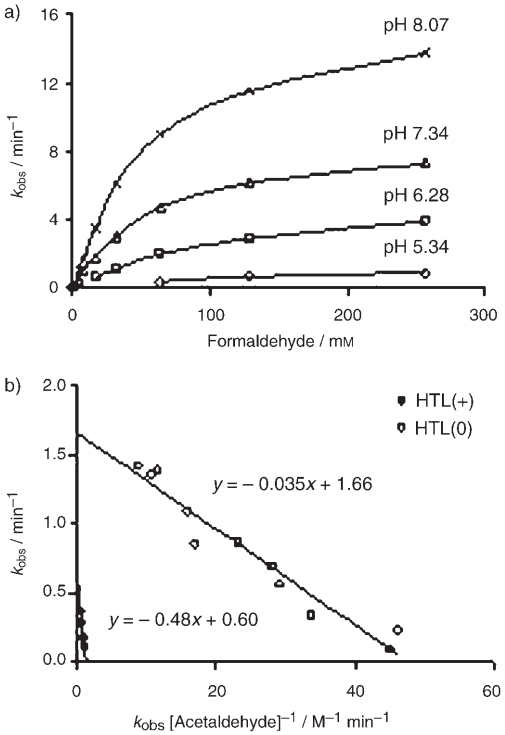


Figure 1. Kinetics of reactions of HTL with formaldehyde (a) and acetaldehyde (b).

There was no catalysis by the phosphate buffer used to maintain pH in the reactions of HTL with formaldehyde or acetaldehyde (not shown). The lack of buffer catalysis is

Table 1. Effect of pH on equilibrium and rate constants for the reactions of HTL with formaldehyde or acetaldehyde.

pH	K_{HCHO} [M]	K_{CH3CHO} [M]	k_{HCHO} [min^{-1}]	k_{CH3CHO} [min^{-1}]
5.34	2.76	2.24	2.36	0.60
5.79		2.48		1.12
6.27	8.55	6.80	5.50	1.26
6.60		10.42		1.78
7.40	15.87	22.73	9.08	1.78
8.01	15.63	21.28	17.70	2.38

somewhat surprising, as an analogous condensation of tetrahydrofolate with formaldehyde is a subject to general acid catalysis by buffers.[26]

Ionization status affects HTL reactivity with aldehydes: The pseudo-first-order rate constants for the reactions of HTL with aldehydes were found to depend on greater than one power of hydroxide ion concentration in the low pH range. At intermediate pH values, the pseudo-first-order rate constants exhibited smaller variations with pH. Plots of the logarithms of apparent third-order-rate constants $k = k_{obs}[aldehyde]^{-1}[OH^-]^{-1}$ versus pH are shown in Figure 2a. For the reaction of HTL with formaldehyde the logarithms of the rate constants follow a sigmoidal curve, with hydroxide-catalyzed segments at low and high pH values and an intermediate region that is less dependent on pH. A similar pH-reactivity profile was obtained for the reaction of HTL with acetaldehyde (Figure 2a). This behavior suggests that HTL undergoes ionization with a pK_a of 6.67 (Scheme 1) and that acid and base species of HTL undergo hydroxide-catalyzed reaction with aldehydes (Figure 2a). The acid form HTL^+ reacts with formaldehyde and acetaldehyde 79 and 73-times faster, respectively, than the base form HTL^0 (Table 2).

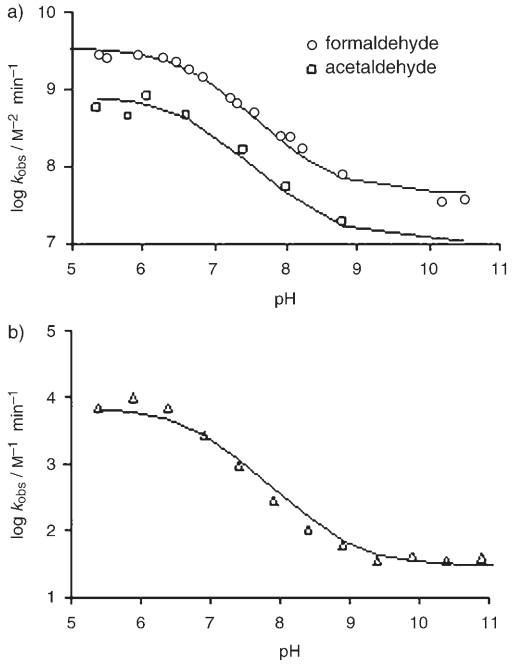


Figure 2. a) pH dependences of the third-order rate constants ($k = k_{obs}[OH^-]^{-1}[aldehyde]^{-1}$) of the reactions of HTL with formaldehyde (○) and acetaldehyde (□). b) pH dependence of the second-order rate constant for the hydrolysis of HTL ($k = k_{obs}[OH^-]^{-1}$) (△). The solid lines are theoretical lines for the reactions of acid and base HTL forms calculated from the equation $k = (k_{HTL}{}^+)(1-\alpha) + (k_{HTL}{}^0)\alpha$ in which α is the fraction of HTL as the base form, based on a pK_a of 6.67.

Table 2. Apparent rate constants for reactions of acid (HTL^+) and base (HTL^0) forms of HTL with aldehydes and hydroxide ion.

Reactants	$k_{HTL}{}^0$	$k_{HTL}{}^+$
HCHO, OH^-	$44\times10^6\,M^{-2}\,min^{-1}$	$3.5\times10^9\,M^{-2}\,min^{-1}$
CH_3CHO, OH^-	$11\times10^6\,M^{-2}\,min^{-1}$	$0.8\times10^9\,M^{-2}\,min^{-1}$
OH^-	$37\,M^{-1}\,min^{-1}$	$6.9\times10^3\,M^{-1}\,min^{-1}$

A similar pH-rate profile was observed for the hydroxide-catalyzed hydrolysis of HTL (Figure 2b), which suggests that ionization status of HTL determines its reactivity in both electorophilic and nucleophilic reactions. The acid form HTL^+ was hydrolyzed 186-times faster than the base form HTL^0 (Table 2). No water-catalyzed reaction of HTL^+ or HTL^0 could be detected.

Comparison of magnitudes of the rate constants (ordinate values in Figure 2a are 10^5-fold greater than ordinate values in Figure 2b) demonstrates that adduct formation between HTL and formaldehyde increases the reactivity of the thioester bond by a factor of 500–1,200 (at 1 mM formaldehyde). Adduct formation between HTL and acetaldehyde increases the reactivity of the thioester bond by a factor of 100–300 (at 1 mM acetaldehyde).

Condensation of HTL with pyridoxal phosphate: The reaction with HTL causes changes in the absorption spectra of pyridoxal phosphate (PLP; Figure 3a) and HTL (not shown). However, the reaction, monitored by changes in absorption at 240 nm (disappearance of HTL) or 390 nm (disappearance of PLP), 450 nm (formation of a PLP-derived imine adduct),[28] and 330 nm (formation of the product PLP-derived tetrahydrothiazine)[29] followed complex kinetics and could not be studied in detail. Initial fast pseudo-first-order kinetics were followed by a process ~10-fold slower. A transient increase in the absorption observed around 450 nm is characteristic of the formation of an intermediate imine adduct.[28] A new absorption peak appearing at 330 nm corresponds to a PLP-derived tetrahydrothiazine.[34]

Like the condensation of HTL with PLP, the condensation of Hcy with PLP also leads to the formation of a new absorption peak at 330 nm, characteristic of PLP-derived tetrahydrothiazine[34] (Figure 3b). The initial drop (at 0.5 min) in absorbance at 390 nm was lower than that observed for the reaction of PLP with HTL, suggesting that Hcy reacts slower than HTL with PLP. However, in contrast to the reaction of HTL with PLP, there was no transient increase in the absorption around 450 nm, which suggests that the reaction of Hcy with PLP occurs by a different mechanism, most likely involving initial formation of hemithioacetal instead of an imine.

HTL ring opening during condensation with aldehydes: The formation of tetrahydrothiazine from HTL would require prior hydrolysis of the thioester bond with the liberation of a free sulfhydryl group. An aldehyde-dependent hydrolysis of HTL was monitored specrophotometrically at 412 nm in the presence of the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Rate constants for formaldehyde- and acetaldehyde-dependent sulhydryl formation (monitored at 412 nm) were essentially identical to the rate constants for HTL disappearance (monitored at 240 nm). However, the amount of the sulfhydryl formed was equal to only 2.3 and 2.5 % of the amount of HTL that disappeared in the presence of formaldehyde and acetaladehyde, respectively. These results suggest that HTL ring opening is slower than the subsequent intramolecular reaction of the liberated thiolate with the aldehyde-derived carbon of the intermediate.

Mechanism of tetrahydrothiazine formation from HTL: The facile formation of tetrahydrothiazines from HTL is surprising and exhibits several unusual features. In contrast to other reactions between amines and carbonyl compounds, which are acid-catalyzed and exhibit bell-shaped pH-rate profiles,[26] the condensation between HTL and aldehydes is hydroxide-catalyzed and its rate increases continuously with pH. The kinetic data for the reaction between HTL and formaldehyde or acetaldehyde fit a model in which the acid form HTL^+ exhibits greater reactivity than the base form HTL^0 with aldehydes (Figure 2a). One would expect that the condensation of HTL with an aldehyde would yield an imine adduct as a final product, as observed in reactions between other amines and aldehydes.[26] However, in the reaction of HTL with formaldehyde or acetaldehyde, imine (which is in a chemical equilibrium with carbinolamine) is formed only as an intermediate, and a final product of the reaction is a tetrahydrothiazine (Scheme 2). The imine intermediate also forms in the reaction of HTL with PLP (Figure 3a). Remarkably, the formation of the carbinolamine intermediate (in chemical equilibrium with the imine,

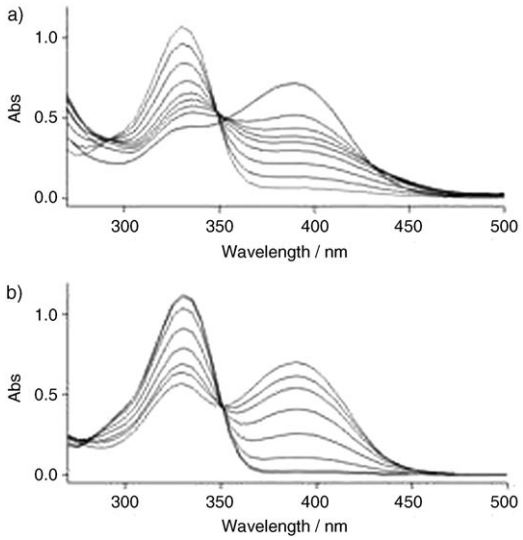
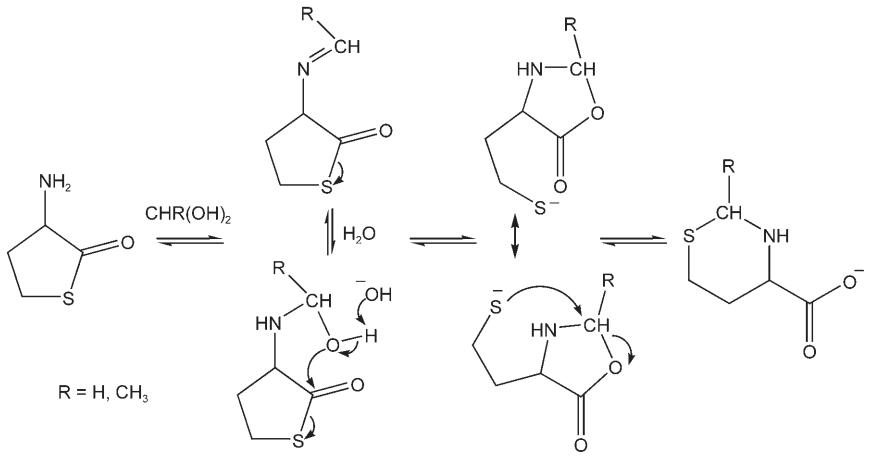


Figure 3. UV-vis absorbance spectra of reactions of HTL (a) and Hcy (b) with PLP. The spectra were taken at 0, 0.5, 1, 2, 4, 8, 16, and 30 min at 24 °C. An additional spectrum was taken for the reaction of HTL with PLP at 60 min. The disappearance of the PLP absorbance at 390 nm is accompanied by the appearance of a PLP-derived tetrahydrothiazine absorbance at 330 nm. Note a transient increase in absorbance around 450 nm (characteristic of a PLP-derived imine) for the reaction of HTL with PLP.

Scheme 2) appears to greatly destabilize the thioester bond. The observed rate accelerations of ~10^4-fold (Figure 2) are most likely caused by an intramolecular anchimeric assistance by the oxygen atom of the carbinolamine intermediate (Scheme 2). Favorable steric configuration facilitates anchimeric assistance by the carbinolamine group, which makes possible an intramolecular attack of the oxygen on the thioester bond to form a five-membered lactone; this leads to the liberation of the thiolate group. Subsequent attack by the thiolate on an aldehyde-derived carbon leads to rapid formation of a six-membered tetrahydrothiazine ring and lysis of the lactone (Scheme 2). This mechanism is reminiscent of the mechanisms proposed for the catalytic acceleration of the hydrolysis of the *p*-nitrophenyl ester of leucine by benzaldehyde,[35] for the rapid hydrolysis of *o*-formylbenzoate esters,[36] and for the hydrolysis of diethylmalonate in the presence of pyridoxal phosphate.[37]



Scheme 2. Proposed mechanism for the condensation of HTL with formaldehyde (R = H) and acetaldehyde (R = CH_3).

Conclusion

The kinetic analysis of facile condensation reactions of HTL with aldehydes and the description of the mechanism involved (Scheme 2) may help to

understand the fate of HTL under physiological conditions. As HTL and aldehydes are ubiquitous, one would expect that corresponding tetrahydrothiazines would also be present in living organisms. However, there is only one report describing the presence of 1,3-tetrahydrothiazine-4-carboxylic acid in a microorganism (*Aerobacter aerogenes*), detected after pulse-labeling with [^{14}C]formaldehyde.[33] The facile formation of tetrahydrothiazines from HTL and aldehydes raises an interesting possibility that the endogenous HTL formed in the human body[6,14,15] may be disposed of in the form of metabolically inactive tetrahydrothiazine. Indeed, in rats injected intraperitoneally with radiolabeled 1,3-tetrahydothiazine-4-carboxylic acid, 55% of the administered compound is excreted in urine and only 6% is expired as carbon dioxide.[32] To what extent the formation of tetrahydrothiazines contributes to metabolic flows of sulfur-containing amino acids in animals and humans remains to be examined.

Experimental Section

General: All chemicals were purchased from Sigma–Aldrich and used without further purification. UV-vis data were obtained by using a Bio50 Varian spectrophotometer at 24 °C.

Kinetics of the reactions of HTL with aldehydes: Reactions, initiated by the addition of homocysteine thiolactone at 24 °C, were followed spectrophotometrically by monitoring the decrease in the absorbance of the thioester bond at 240 nm ($\varepsilon = 5000\,\text{M}^{-1}\text{cm}^{-1}$)[13] in a 1 or 0.2 cm light path length cuvette. Reaction mixtures (0.5 mL) contained sodium-phosphate buffer (0.1 M), HTL (0.1–2.5 mM, as hydrochloride), and aldehyde (2–250 mM). Reactions, followed pseudo-first-order kinetics for up to 5 half-lives. Reactions were found to go to completion and the end points were stable. The values of pseudo-first-order rate constants, k_{obs}, were calculated by using Varian Bio50 software. No catalysis by the phosphate buffer (0.05–0.5 M) was observed.

Aldehyde-dependent HTL ring opening: Reaction mixtures (0.5 mL) contained sodium-phosphate buffer (0.1 M , pH 7.4), HTL (0.4–2.5 mM, as hydrochloride), formaldehyde or acetaldehyde (4–10 mM), and DTNB (0.4 mM). The reactions were initiated by the addition of an aldehyde. Ring opening and HTL disappearance were monitored at 412 and 240 nm, respectively, by using a 0.2 cm light path length cuvette.

pK_a determinations: The pK_a of HTL was determined at 24 °C by potentiometric titration of the hydrochloride salt of HTL (2.5–33.3 mM) with NaOH. pH was monitored by using a Beckman Φ310 pH meter. The measurements gave a mean value of $\text{p}K_a = 6.67 \pm 0.05$ from eight independent runs on three separate occasions.

Acknowledgements

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