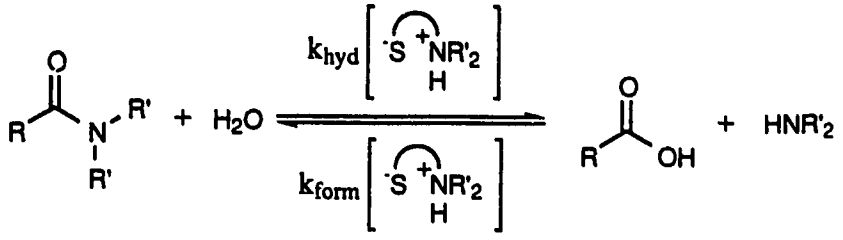
formation of that amide from its constituent acid and amine (k_{form} from **Scheme 24**). The pursuit of this possibility is described in Chapter 3.

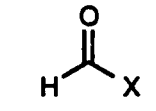
Scheme 24



CHAPTER 3

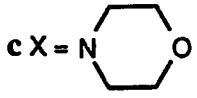
A) Introduction

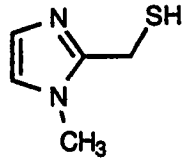
As discussed in Chapters 1 and 2, previous work in this laboratory[50b,54] has shown that the zwitterionic form of **7b** reacts rapidly with pNPA and with distorted anilide **2** to yield the corresponding thiolesters. These thiolesters then hydrolyze in aqueous solution with the assistance of the pendant imidazole to produce the respective carboxylic acids thereby regenerating the thiolate in its active form. Described in this chapter is the use of 2-mercaptomethyl-N-methylimidazole (**7b**) as a catalyst for the hydrolysis of the non-activated amides formamide, dimethylformamide (DMF), and N-formylmorpholine (NFM), amides **20a-c**. Also reported herein is the use of **7b** as a catalyst for the microscopic reverse reaction, namely the re-formation of **20c** from its constituent acid and amine in D_2O at near neutral pD values.[55]



20a X = NH_2

b X = $N(CH_3)_2$

c X = 



7b

B) Experimental

a) General: High field NMR spectra were recorded on a Bruker WH-200 (200 MHz) spectrometer.

b) Materials: Formamide (2[illegible] .N-dimethylformamide (DMF, **20b**), N-formyl-morpholine (NFM, **20c**) and sodium 3-trimethylsilylpropane sulfonate (DSS) were commercially available (Aldrich) and were used without further purification. Phosphate buffer (KH_2PO_4 / K_2HPO_4) was reagent grade (Sigma) and used as supplied. Deuterium oxide (99.9 %, Aldrich) was purged of O_2 by passing a stream of Ar through it for several hours. 2-Mercaptomethyl-N-methylimidazole (**7b**) was prepared as reported.[50b]

c) Kinetics: In a typical kinetics experiment, a solution 100 mM (for DMF or formamide) or 200 mM (for NFM) in amide, 0 - 150 mM in **7b**, and 35 mM in sodium 3-trimethylsilylpropane sulfonate (DSS) as a ^1H NMR standard was prepared in 6 - 10 mL of D_2O at pD 7.8 - 8.0 (buffered by **7b** itself) with the ionic strength controlled by KCl such that μ=1.0. Aliquots of this solution 0.6 mL in volume were then transferred into each of 8 argon-purged 5 mm NMR tubes, which were flame-sealed. ^1H NMR spectroscopy was performed on two of the tubes for the "zero time" spectra. All of the tubes were then heated to 98 °C (refluxing water vapour) and at various times, duplicate tubes were removed and frozen. The ^1H NMR spectra of all eight tubes were subsequently recorded, and the ^1H NMR signals due to the formate-H (δ 8.5) and formyl-H (δ 8.0) were integrated relative to DSS to determine the progress of the hydrolysis reaction. The solution pD was measured before and after the reactions with a Radiometer VIT90 Titrator equipped with a GK2401C Combination Electrode and did not change by more than 0.05 units.

For formamide, the hydrolysis was sufficiently fast so that a non-linear least squares (NLLSQ) fit of the exponential decay of formamide concentration with time was possible, giving a pseudo-first order rate constant for each concentration of **7b**. However,

for the slower hydrolyses of DMF and NFM, the initial rates of the reactions were determined by measuring the linear least squares slope of the first 6 - 10 points on the plots of amide concentration versus time for the initial 3 - 10% of the reaction (up to 14 days required for the blank runs) and dividing by the initial amide concentration. This was necessary, as the catalyst appeared to begin to decompose after ~24 hours, to what is believed to be a product of oxidation, such as the corresponding disulfide.

d) Formation Reactions: The formation of NFM was similarly followed by ^1H NMR in D_2O, pD 8.0, μ = 1.0 (KCl), using solutions 0 or 100 mM in **7b**, or 200 mM in phosphate, and 200 or 400 mM in both formic acid and morpholine. The observed pseudo-first order rate constants were determined by dividing the initial slope (3 - 5 points, in duplicate) of the plot of [NFM] vs time by the initial concentration of formic acid (200 mM). For the blank reaction, 7 days were required to generate 5% reaction. The second order rate constant was determined by measuring the slope of the plot of the pseudo-first order rate constants of formation of NFM versus the concentration of **7b** (0 - 100 mM, with an additional point at 100 mM [**7b**] derived from half the rate observed at 400 mM formic acid).

e) Equilibrium Endpoint Determination: Two sealed NMR tubes 200 mM in each of formic acid, morpholine, and phosphate (pD 7.6, μ = 1.0 (KCl)) were heated for 20 days at 98 °C. Periodically, the tubes were removed from heating, cooled, and examined by ^1H NMR. Non-linear least squares analysis of the 8 data points of [NFM] vs time so collected gave a pseudo-first order rate constant for formation of NFM with phosphate catalysis. The hydrolysis of 200 mM NFM in the presence of 100 mM phosphate was monitored in an identical manner to give a pseudo-first order rate constant for the hydrolysis of NFM with phosphate catalysis. The final, asymptotic value of [NFM] from the exponential fit for both of these experiments was identical within experimental error.

C) Results and Discussion

a) Hydrolysis: The kinetics of the reactions of the various amides with **7b** were followed at pD 7.8 - 8.0 at 98 °C and μ = 1.0 (KCl). At these pD values, it has been shown that at 25 °C thiolimidazole **7b** exists predominantly as a zwitterion[50b] in an aqueous environment. Although the pKa values of both the thiol and imidazolium groups are anticipated to drop by about 0.6 units (according to pKa = ΔG° / (2.303 RT)) on the change in temperature from 25 to 98 °C, the zwitterionic form of thiolimidazole **7b** is still expected to be the dominant form in solution, and as such is in its most catalytically active form.[50b,54] ^{1}H NMR signals due to the formate-H (δ 8.5) and formyl-H (δ 8.0) of substituted formamides **3a-c** were integrated relative to sodium 3-trimethylsilylpropane-sulfonate (DSS) to determine the progress of the hydrolysis reaction. The pseudo-first order rate constants (see Appendix 3) were then plotted against catalyst concentration for each amide, giving the observed second order rate constants reported in **Table 4**. The observed pseudo-first order rate constants for the blank, or uncatalyzed reactions for these amides at the indicated pD are also given in **Table 4**. In no case is the blank reaction negligible. No accumulation of thiolester **21** was observed in any case, indicating its concentration must be in a steady state, and that **7b** is acting as a true turnover catalyst. There seems to be no justifiable reason to invoke a mechanism for this catalytic hydrolysis other than the one outlined in the preceding chapters, represented in **Scheme 25**.

b) Amide Formation: There is literature precedence (both theoretical[84] and experimental[84,85]) to suggest that at neutral pH, an amide can exist in equilibrium with its hydrolysis products. For most alkyl amine amides, the position of this equilibrium - the endpoint of the hydrolysis reaction - lies so far to the hydrolysis products that the existence of such an equilibrium becomes difficult to detect. However, Fersht has indirectly determined[85] an equilibrium constant[87] for the formation of N-formylmorpholine (see

equation (**10**)) of 1.35 M^{-1} (at pD 7.7), a value that indicates a significant amount of acid, amine and amide would be present at the endpoint under conditions employed herein.

Table 4 : Rate Constants for the Hydrolysis (and Formation) of Various Formamides Mediated by Thiolimidazole **7b** in D_2O Solution (T = 98 °C, μ = 1.0 (KCl))

Amide	$k_2^{obsd} \times 10^5$ ($M^{-1}s^{-1}$)[a]	$k_0^{obsd} \times 10^8$ (s^{-1})[f]
20a	13 ± 1[b]	36.4 ± 4.2[g]
20b	1.6 ± 0.2[c]	2.8 ± 0.6[g]
20c	1.1 ± 0.1[d]	3.0 ± 0.1[c]
(**20c** formation)	((3.9 ± 2.3) × 10^{-2})[e]	

[a] The second order rate constants are the slopes of the linear least squares fits of the plots of the pseudo-first order rate constants for hydrolysis vs four concentrations of **7b**, determined in duplicate. The reported errors are the standard deviations of the linear least squares treatment. Due to the inherent difficulties in obtaining kinetic rate constants from NMR data, real errors are estimated to be ± 10%.

[b] pD 7.8.

[c] pD 8.0.

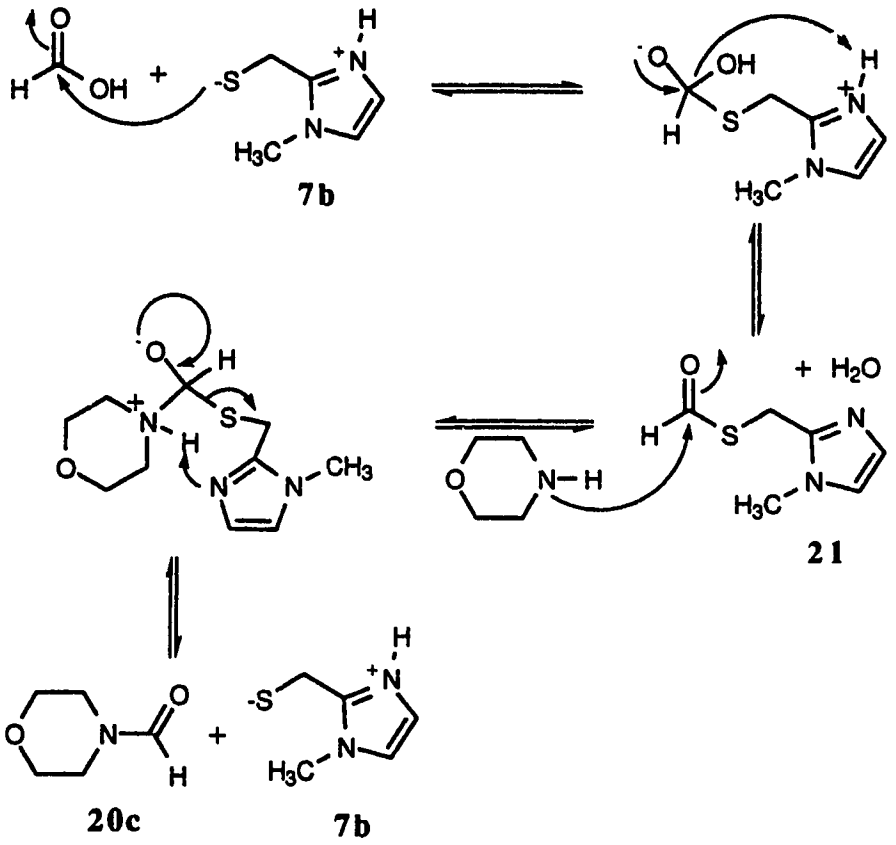
[d] k_{cat} of **Scheme 26**, pD 8.0.

[e] k_{dehyd} of **Scheme 26** was determined as the slope of the linear least squares fit of the plot of k^{obsd} for the formation of NFM vs [**7b**] at 200 mM [formic acid] (pD 7.7, see **Table 18S**, Appendix 3).

[f] Observed pseudo-first order rate constants measured for hydrolysis with no added catalyst. Determined by the initial rate method over more than two weeks.

[g] pD 7.9.

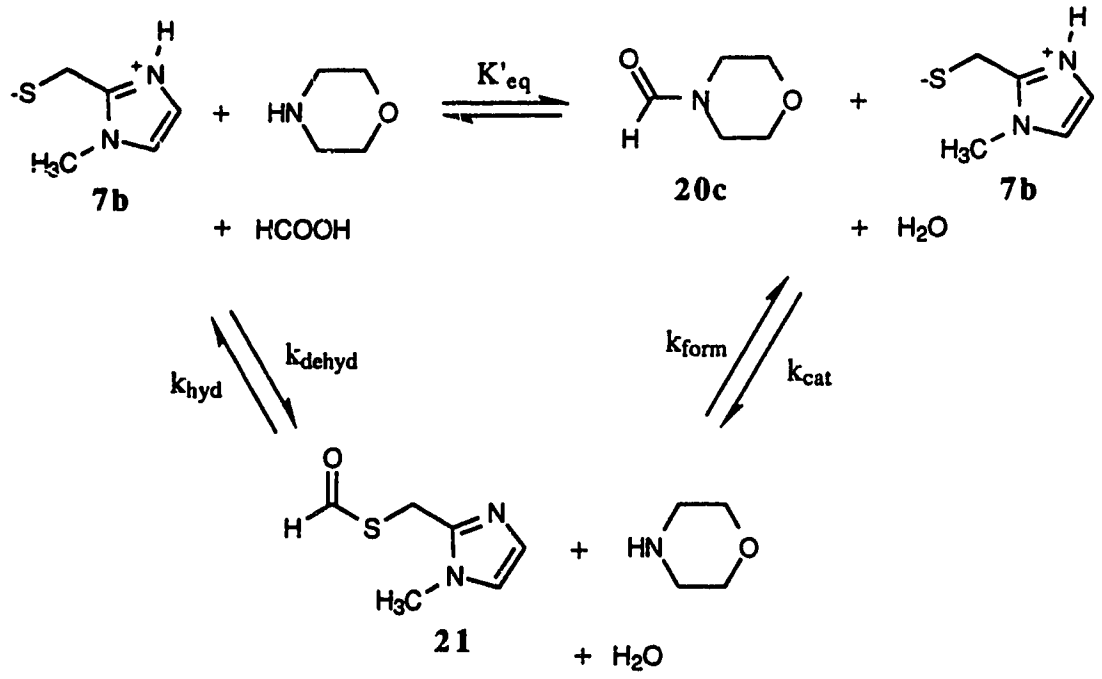
Scheme 25



The formation of NFM from formic acid and morpholine was similarly followed by ^1H NMR in D_2O, using **7b** or phosphate as a buffer and catalyst.[88] From the observed pseudo-first order rate constants obtained using 0 - 100 mM **7b** as a catalyst (see Appendix 3), it was possible to determine a value for the observed second order rate constant for the catalyzed formation of NFM of $(3.9 \pm 2.3) \times 10^{-7}$ $M^{-1}s^{-1}$. Thiolester **21** (see **Scheme 25**) was not observed to accumulate in the formation of NFM under the conditions employed, indicating the concentration of thiolester was again in a steady state. When the concentrations of formic acid and morpholine were both doubled, the rate approximately doubled (within experimental error) but did not quadruple, indicating that only formic acid is involved in the rate determining step, which is probably the formation of the intermediate

thiolester. A catalytic cycle consistent with all of the observed data is shown in **Scheme 26**.

Scheme 26



$$K'_{eq} = \frac{k_{dehyd}}{k_{hyd}} \times \frac{k_{form}}{k_{cat}} = \frac{k_{dehyd}}{k_{cat}} \times \frac{k_{form}}{k_{hyd}} = 1.2 \pm 0.2\ M^{-1}$$

The detailed mechanism for this thiolester formation is believed to be simply the microscopic reverse of the mechanism for thiolester hydrolysis described in Chapter 2. That is (see **Scheme 25**), attack of **7b** on formic acid is followed by intramolecular imidazolium general acid catalyzed breakdown of the tetrahedral intermediate with loss of a water molecule to give the formyl thiolester. Rapid intermolecular aminolysis of this thiolester through nucleophilic attack of the morpholine nitrogen then produces a second

tetrahedral intermediate, from which the imidazolium thiolate is ejected as an excellent leaving group, in its catalytically active form.

Using the steady state approximation, (steady state in [thiolester **21**]) the rate of amide formation can be expressed by equation (**6**) (see Appendix 3 for derivation). Since the rate of amide formation was found to be roughly independent[89] of morpholine concentration, the partitioning of the thiolester intermediate of **Scheme 26** must be such that, under the conditions of this study, $k_{form}[amine] > k_{hyd}$. As is shown in equation (**7**), the observed rate constant for amide formation is therefore k_{dehyd}.

$$V_f = \frac{k_{dehyd}\, k_{form}[acid][cat][amine]}{k_{form}[amine] + k_{hyd}} \qquad (6)$$

$$V_f = k_{dehyd}[acid][cat] \qquad (7)$$

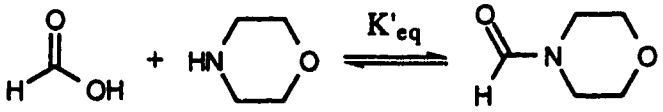
Similarly, an expression for the rate of the hydrolysis reaction can be derived (see Appendix 3) and is given in equation (**8**). Under the conditions employed here, the concentration of amine present during the hydrolysis of amide is vanishingly small, so that equation (**8**) reduces to equation (**9**) and the observed rate constant of hydrolysis is k_{cat}.

$$V_r = \frac{k_{hyd}\, k_{cat}[amide][cat]}{k_{form}[amine] + k_{hyd}} \qquad (8)$$

$$V_r = k_{cat}[amide][cat] \qquad (9)$$

c) Equilibrium Endpoint: When the reactions of NFM hydrolysis and formation were allowed to proceed to "completion" as shown in **Figure 7**, (~6 half-lives, using phosphate as a catalyst[88]) the equilibrium position for the formation of NFM from formic acid and morpholine was determined by the endpoint of the reaction (33 mM NFM, 167 mM formic acid, 167 mM morpholine). This allowed calculation of K'_{eq}, as defined in

equation (**10**). Our so determined experimental value for K'_{eq} of 1.2 ± 0.2 M^{-1} compares favourably to the value of 1.35 M^{-1} reported by Fersht.[85,87]

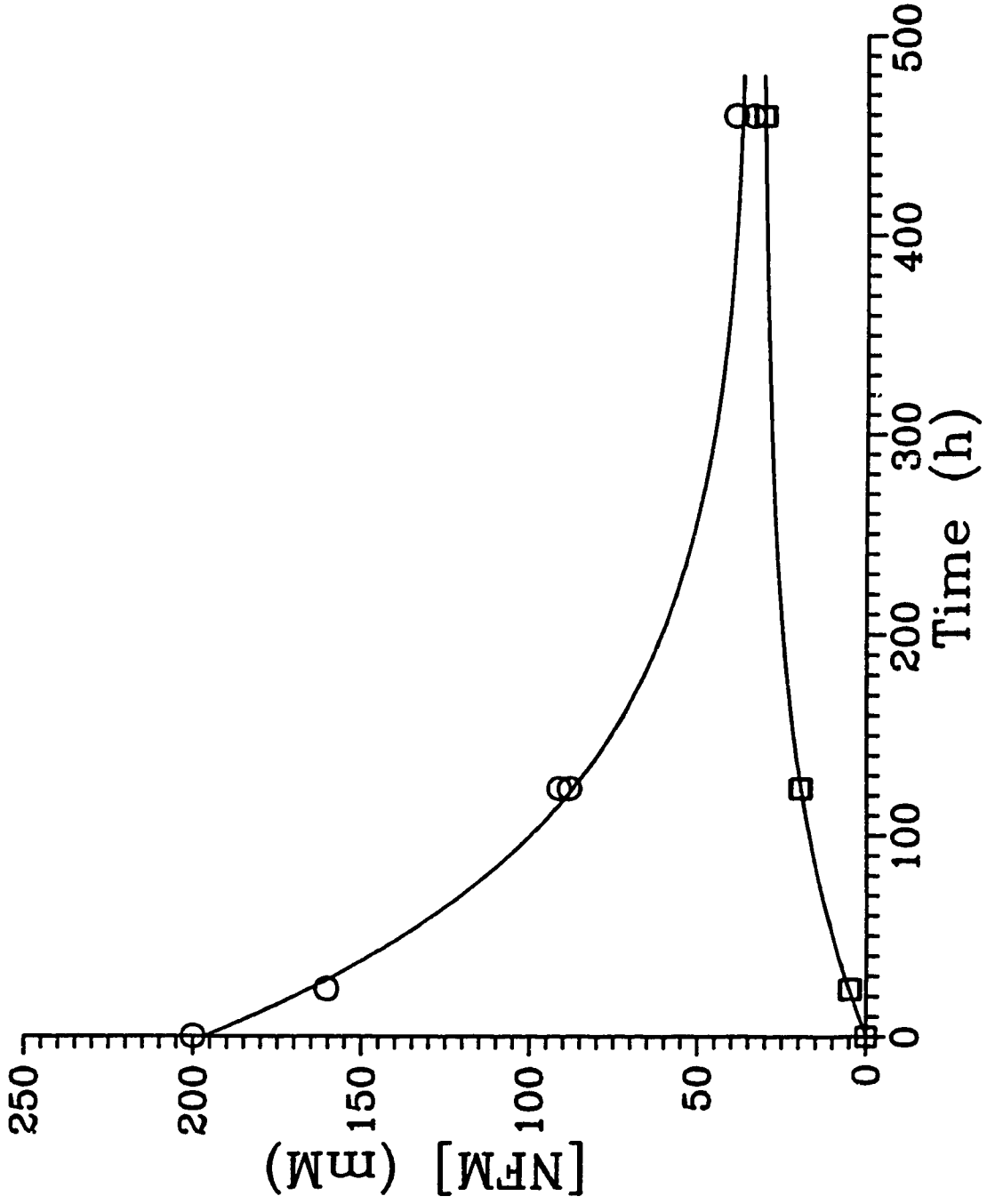


(**10**)

$$K'_{eq} = [NFM] / ([HCOOH]_{tot}\,[morpholine]_{tot})$$

Finally, since the thermodynamic cycle can be drawn as in **Scheme 26**, and the values of K'_{eq}, k_{cat} and k_{dehyd} can be measured (see **Table 4**, footnotes d and e for k_{cat} and k_{dehyd}, respectively) a value can be determined for the ratio of k_{hyd} to k_{form}. This partitioning ratio of the intermediate thiolester between acyl transfer to amine (k_{form} for the reaction with morpholine) and hydrolysis (k_{hyd} for the reaction with water) was determined to be $k_{hyd}/k_{form} = (3.0 \pm 1.5) \times 10^{-2}$ M. This ratio corresponds to the concentration of free morpholine that must be present for the thiolester intermediate to partition equally between hydrolysis and aminolysis.

Figure 7 : Establishment of equilibrium endpoint between NFM hydrolysis (circles, $k^{obsd} = (2.5 \pm 0.2) \times 10^{-6}\ s^{-1}$) and formation (squares, $k^{obsd} = (2.2 \pm 0.1) \times 10^{-6} s^{-1}$) in D_2O; T = 98 °C, pD 7.6, μ=1.0 (KCl), [phosphate] = 200 mM (see reference 88). Solid line is the non-linear least squares fit of the data. $[NFM]_{eq}$ was found to be 33 ± 2 mM.



D) Conclusions

There are three salient points suggested by this work:

1. It has been clearly shown that under the reaction conditions employed, the hydrolysis of N-formylmorpholine is reversible (see equation (**10**)) and does not proceed to completion but rather to an equilibrium position. This equilibrium position has also been approached from the reverse direction in the formation of the amide from its constituent acid and amine. This modest catalysis by thiolimidazole **7b** of the formation of an unstrained amide from its constituent carboxylic acid and alkyl amine serves as a phenomenological model for peptide formation mediated by the cysteine proteases.

2. 2-Mercaptomethyl-N-methylimidazole (**7b**) is one of the first man-made non-metal-containing turnover catalysts of the hydrolysis of unactivated amides at physiological pH and elevated temperature. By way of comparison, the second order rate constants reported herein are within an order of magnitude of those reported recently by Chin[90] for a metal-containing catalyst. However, since this simple model system lacks the obvious catalytic advantages enjoyed by the cysteine proteases attributable to the correct orientation of the catalytically essential residues and the desolvation of substrates within the active site, this enzyme model is not as effective a catalyst as the enzyme itself.

3. There is an observable blank amide hydrolysis reaction (independent of catalyst) active in the neutral pH region that makes a non-negligible contribution to the rate of hydrolysis of amides mediated by poor catalysts. This reaction is slow, but several times faster than expected from extrapolation of the second order acid and base hydrolysis terms[91] to neutral pH, indicating the presence of an additional significant water term.[92]

CONCLUSION

A) Relevance to the Cysteine Proteases

a) Structure: The small bifunctional nucleophile. 2-mercaptomethyl-N-methylimidazole (**7b**) is incapable of modeling the binding site of an enzyme, but by incorporating an imidazole ring and an adjacent thiol group intramolecularly it approximately models the essential histidine and cysteine residues[32] in the active site of the cysteine proteases. At pH levels near neutrality, at which the enzymes display maximum activity, **7b** has previously been shown[50] to exist in aqueous solution predominantly as a zwitterion. In this way, **7b** may approximate the zwitterionic ion pair believed[37,38] to exist in the active site of the enzyme at physiological pH.

b) Acylation: During the enzyme mediated hydrolysis of amides, the acylation of the active site thiolate of many cysteine proteases appears to be rate limiting.[34] For typical amide substrates this acylation step follows a bell-shaped pH/rate profile consistent with the kinetic competence of a neutral or more probably the zwitterionic form of the active site, in which an essential sulfhydryl residue is ionized (pKa ~ 3.9 - 4.3) and an essential imidazole residue is protonated (pKa ~ 8.2 - 8.5).[35] The reaction of **7b** with strained amide **2** displays an analogous bell-shaped pH/rate profile, demonstrating herein the essentiality of the pendant imidazolium ion to the acylation of the thiolate. Furthermore, this model system has revealed (Chapter 1) the role of this imidazolium to be that of an intramolecular general acid trap of the unstable tetrahedral intermediate formed through the nucleophilic attack of thiolate on the anilide carbonyl. In the acylation of papain with amides,[35c] the poorness of the amino leaving group causes the breakdown of a similar tetrahedral intermediate to become rate limiting, and to require general acid assistance. The model system presented here provides chemical precedence for the involvement of the histidine imidazolium as this general acid trap, as shown in the Introduction in **Scheme 7**.

c) Deacylation: The deacylation step of the cysteine proteases is thought to proceed through general base assisted hydrolysis of the acyl-enzyme thiolester. The observed apparent pKa[31a,35a] and skie values[35a,39] for this process are consistent with the histidine imidazole acting as the general base.[33i,40] Earlier model systems of pendant amino thiolester hydrolysis (wherein the amino and thiol groups were retained intramolecularly[50]) clearly demonstrated the general base role of the pendant amino group. As shown in Chapter 2, the hydrolysis of thiolesters **13b** and **17** is believed to proceed through a similar involvement of the amino groups but there are structural components to these that introduce significant competing processes. Although the clarity of a mechanism showing the nature of the general base process was clouded by intramolecular aminolysis of these thiolesters, a general base role is consistent with the evidence herein. On the basis of this model study and those conducted earlier in this laboratory,[50] the deacylation step of the cysteine proteases involves general base catalysis and requires no S→N acyl transfer. This deacylation mechanism is also represented in **Scheme 7** in the introduction.

d) Catalysis: The system examined herein is able to model both the acylation and deacylation of the cysteine proteases by amides through its intramolecular incorporation of a thiolate and imidazolium group. As the zwitterionic thiolate **7b** attacks the amidic carbonyl of **2**, the adjacent imidazolium is able to act as a general acid trap of the tetrahedral intermediate, preventing the expulsion of the thiolate and taking the intermediate on to thiolester formation (see **Scheme 18** of Chapter 1). This thiolester (**17**) is generated with a pendant imidazole that can now act as a general base in the assistance of the hydrolysis of the thiolester. A product of this hydrolysis is the imidazolium thiolate **7b**, regenerated in its active form. As mentioned in the Introduction, the appeal of the application of these model mechanisms to cysteine protease catalyzed amide hydrolysis is found in the elegance with which each successive step is accomplished. Each step generates as a product of that step the form of the catalyst/enzyme required to mediate the next transformation.

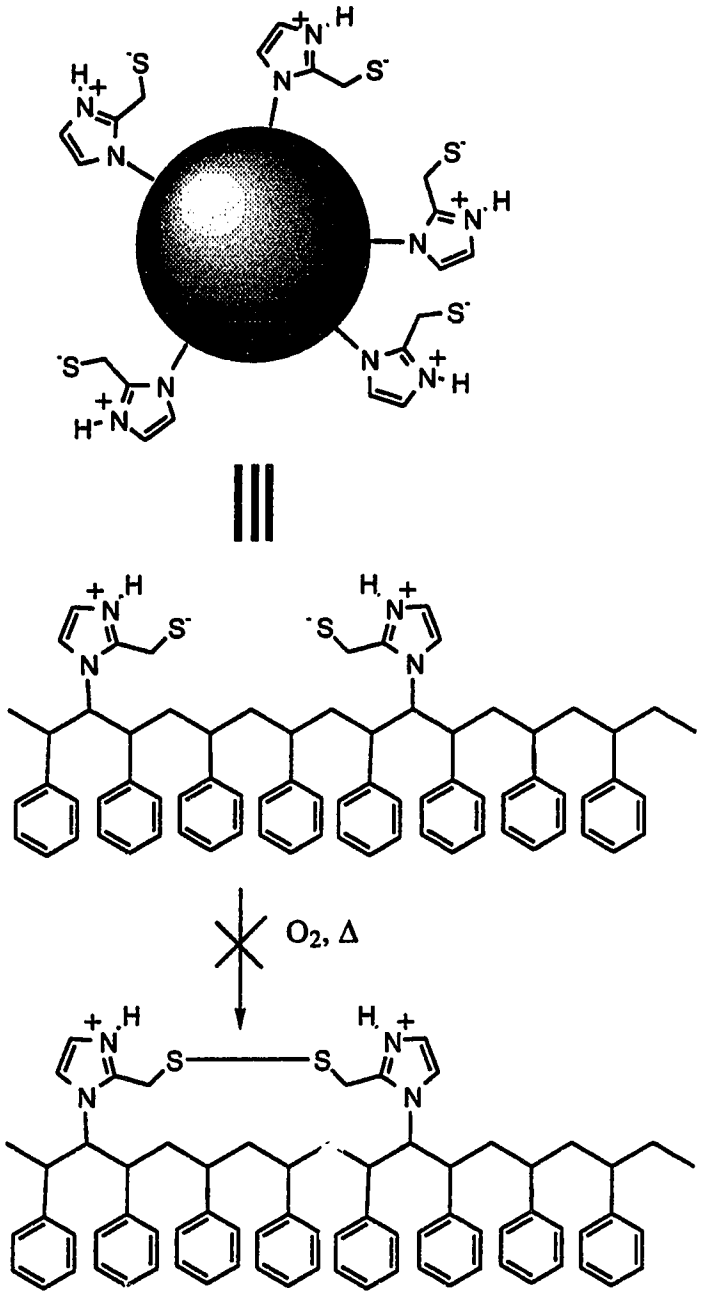
B) Future Considerations

Although the reaction of thiolamines with a strained amide has served as a good model for the acylation and deacylation steps of the cysteine proteases, the portion of this work that holds the most promise for intriguing future studies is the catalytic hydrolysis and formation of unstrained amides, discussed in Chapter 3. Generally speaking, a catalyst of amide hydrolysis must contain both a nucleophile and an intramolecular proton source (general acid trap). Evidence for this is found in the mechanism of amide hydrolysis followed by all three classes of proteolytic enzymes and several model systems discussed in the Introduction. Catalysis of the microscopic reverse reaction - the formation of an amide from its constituent carboxylic acid and amine - must hold the same requirements.

In this study, the best catalyst of both of these processes was found to be thiolamine **7b**. As a zwitterion in solution, it contains a nucleophilic thiolate and an acidic imidazolium in close proximity to each other. The hydrolysis of strained amide **2** and unstrained amides **20a-c** as well as the formation of amide **20c** were accelerated by the presence of **7b**. While these results are encouraging for further investigation, the oxidative and thermal instability of the model compound have been noted as an obstacle to more widespread use of **7b** as a general catalyst for these transformations.

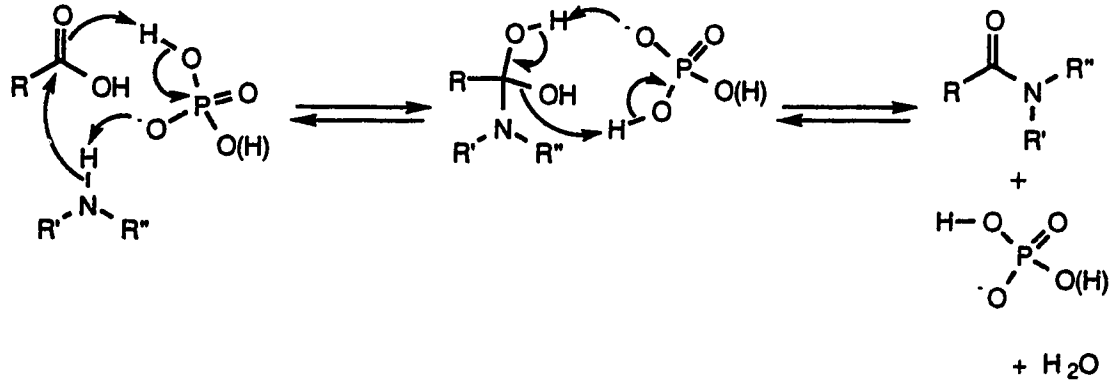
One possibility for future stabilization of this thiol which is currently being investigated in this laboratory is the covalent attachment of **7b** to a solid support, as shown in **Scheme 27**. If the distance between thiolate groups is sufficiently large to prevent oxidative disulfide formation, then the lifetime of the catalyst in its active form may be extended. Another advantage to the construction of this catalyst is the ease of handling associated with the use of polystyrene beads as the solid support. It is envisioned that these beads could be added to a solution of amide, the solution heated to reflux, and the amide hydrolysed. After the reaction was complete, the beads could simply be filtered away from the solution, ready to use again.

Scheme 27



Another catalyst of both amide hydrolysis and formation that was discovered through this study is phosphate (KH_2PO_4 / K_2HPO_4). Although phosphate is not as active a catalyst as **7b** under the conditions employed in this work, it has been demonstrated that phosphate shows an advantage over **7b** in its geater stability to heating for prolonged times. This ability of a common, naturally occurring inorganic molecule to catalyze the formation of amides (possibly including peptides) is of great interest. Currently, work is underway in this laboratory to attempt the catalytic formation of small dipeptides and to investigate the mechanism of catalysis by phosphate. Two possible mechanisms envisioned for the observed catalysis of amide formation by phosphate are the general base / general acid catalysis pathway and the bifunctional catalysis pathway. Bifunctional catalysis has been invoked to account for the accelerated rate of amide[93] and imidate ester[94] hydrolysis as well as amide thiolysis[73] by buffers such as phosphate, acetate, carbonate and arsenate. These buffers are able to mediate acyl transfer reactions through concerted proton addition and removal in the formation and breakdown of tetrahedral intermediates. The mechanism of bifunctional catalysis of amide hydrolysis by phosphate is presented hypothetically in **Scheme 28.**

Scheme 28



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3. The derivation leading to the kinetic expressions is straightforward. If k_1 is redefined to include the protonation, and k_2 is redefined as including the equilibrium between T^0 and T_N+ and the subsequent breakdown step, then the rate constants can be defined as

   $$k_{hyd} = k_1k_2/(k_{-1} + k_2)$$

   and $$k_{ex} = k_1k_{-1}/2(k_{-1} + k_2)$$

   such that $$k_{ex}/k_{hyd} = k_{-1}/2k_2.$$

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$$k_{hyd} = k_1k_2[OH^-] / (k_{-1} + k_2)$$

and $$k_{ex} = k_1k_{-1}[OH^-] / 2(k_{-1} + k_2).$$

When exchange is small relative to hydrolysis, $k_2 >> k_{-1}$, so

$$k_{hyd} = k_1[OH^-]$$

and $$k_{ex} = k_1k_{-1}[OH^-] / 2k_2$$

such that $$k_{ex}/k_{hyd} = k_{-1} / 2k_2.$$

Similarly, if hydrolysis is much slower than exchange, $k_2 << k_{-1}$ so that

$$k_{hyd} = k_1k_2[OH^-] / k_{-1}$$

and $$k_{ex} = k_1[OH^-] / 2$$

such that $$k_{ex}/k_{hyd} = k_{-1} / 2k_2,$$ again.

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68. The pKa values quoted in **Scheme 17** are the correct ones - those reported in reference 50b, Table IV for 2-thiomethylimidazole, and N-methyl-2-thiomethyl-imidazole should be interchanged.

69. The actual equation fit was:

$$k_2^{obsd} = (k_2 K_{ZW}) [H^+] / (([H^+]^2 / Ka^{Im}) + (1 + K_{ZW}) [H^+] + Ka^{SH})$$

70. The macroscopic Ka values are defined as:

$Ka^1 = Ka^{Im} + K_1$ and $1/K_a^2 = 1/Ka^{SH} + 1/K_3$

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87. This apparent equilibrium constant takes into account the amount of formic acid and morpholine that would exist as their neutral species at pH levels between the pKa values of formic acid and morpholinium ion, so that the total concentrations of formic acid and morpholine may be used in the equilibrium expression :

$K_{eq} = 2.24 \times 10^5\ M^{-1} = [NFM] / [HCOOH]\ [morpholine]$;

$K'_{eq} = 1.35\ M^{-1} = [NFM] / [HCOOH]_{tot}\ [morpholine]_{tot}$.

See Appendix 3 for derivation.

88. Phosphate buffer (i.e. KH_2PO_4 / K_2HPO_4) was serendipitously found to have a modest catalytic effect on the hydrolysis of the three amides studied, with the advantage over **7b** of being more thermally stable over long periods of heating.

89. On doubling the concentration of both morpholine and formic acid, the observed rate of amide formation actually increased by ~ 240%. The partitioning ratio of k_{hyd}/k_{form} was determined to be 30 mM (*vide infra*). Using this value, the ratio of $(k_{form}[amine])/k_{hyd}$ can be calculated to be 6.6 at 200 mM [morpholine] and 13.2 at 400 mM [morpholine]. The approximation that $k_{form}[amine] + k_{hyd} \approx k_{form}[amine]$ may partially account for the additional 40% rate increase. However, we believe the assumption that the rate of formation of NFM is independent of morpholine concentration under these conditions to be justified in this approximate treatment of the data.

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APPENDIX 1

Supplementary Material to Chapter 1

Preparation of Amide **14a**:

0.6 g (3 mmol) of [2-(benzylthio)ethyl]amine-HCl was dissolved in 2.5 equivalents (0.7 g, 1 mL) of triethylamine and 25 mL $CHCl_3$. Into this solution was dripped, with stirring, a $CHCl_3$ solution of 1 equivalent of the respective N-tosylated acid chloride of **2**. Stirring was continued for two hours, followed by extraction of the $CHCl_3$ once with 0.5 M HCl and twice with H_2O. The aqueous layers were extracted once again with $CHCl_3$, the organic layers combined, dried over $MgSO_4$, and stripped of solvent. A syrup was obtained in 95% yield and was characterized by 80 MHz NMR, FTIR, and EI mass spectroscopy. The syrup was purified through silica gel column chromatography (20/80 EtOAc/pet. ether) and 0.45 g of the material was added to a flask fitted with a dry ice condenser and dissolved in 7 mL freshly distilled THF. The flask was cooled to -50 °C in a dry ice/acetone bath and 80 mL of $NH_{3(l)}$ was condensed inside. Sodium metal was added until a blue colour persisted, stirring was continued for 15 minutes, and ammonium chloride was added until the solution turned yellow. The condenser and bath were removed and the ammonia was allowed to evaporate under gentle Ar flow. The remaining powder was dissolved in Ar-purged EtOH, and the solution filtered of salts. Bubbling HCl gas through the solution precipitated out some more salts, which were also removed through filtration. The solvent was then removed, leaving behind a yellow residue, which was dissolved in Ar-purged H_2O, adjusted to pH 7 (with 1 M NaOH) and extracted with Ar-purged $CHCl_3$. The organic layers were combined, dried over $MgSO_4$, and removed. The waxy solid (**14a**) was purified through preparative reversed-phase TLC (14/86 H_2O/MeOH) and characterized : ^1H NMR (300 MHz, $CDCl_3$) δ 6.95 (m, 2H), 6.60 (d of t, 1H), 6.45 (d of d, 1H), 6.34 (s), 3.8 (s, 1H), 3.54 (q, 2H), 3.28 (q, 2H), 2.97 (t, <

1H), 2.76 (t, 2H), 2.30 (m, 1H), 2.25 (m, 2H), 2.09-1.84 (br m, 2H), 1.84-1.67 (br m, 2H), 1.4-1.0 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) 173.54, 4° C; 144.22, 4° C; 129.13; 3° C, 127.05, 3° C; 124.04, 3° C; 116.54, 3° C; 114.16, 3° C; 77.47, 77.04, 76.62, ($CDCl_3$ triplet); 38.43, 2° C; 38.27, 2° C; 37.76, 2° C; 35.08, 3° C; 33.89, 2° C; 31.90, 2° C; 26.22, 2° C; FTIR ($CHCl_3$, cm^{-1}) 3307, 3002, 2936, 1647, 1605; MS (NH_4^+ positive ionization) m/z (relative intensity) 265 ($M-H^+$, 100); 261 (15.5); 247 (10.5); 234 (15.9); 188 (4.5).

Tables of Observed Second-order Rate Constants:

Table 1S : Second Order Rate Constants at Various pH Values for the Reaction of Cysteamine (**10a**) with Distorted Amide **2** (T = 25 °C, μ = 1.0 (KCl))

pH	k_2^{obsd} ($M^{-1}s^{-1}$)[a]
6.15	0.962 ± 0.044
7.25	7.03 ± 0.23
8.05	34.2 ± 1.5
8.65	62.7 ± 1.8
9.35	90.8 ± 0.8

[a] Relative error calculated from the standard deviation of the fit of k_{obsd} vs $[RSH]_{total}$ at the given pH level.

Table 2S : Second Order Rate Constants at Various pH Values for the Reaction of N,N-Dimethylaminoethanethiol (**10b**) with Distorted Amide **2** (T = 25 °C, μ = 1.0 (KCl))

pH	k_2^{obsd} ($M^{-1}s^{-1}$)[a]
6.25	0.929 ± 0.013
7.15	5.17 ± 0.05
7.98	20.3 ± 0.5
8.39	25.3 ± 0.7
9.33	29.3 ± 1.0

[a] Relative error calculated from the standard deviation of the fit of k_{obsd} vs $[RSH]_{total}$ at the given pH level.

Table 3S : Second Order Rate Constants at Various pH Values for the Reaction of 4-(2-Mercaptoethyl)morpholine (**10c**) with Distorted Amide **2** (T = 25 °C, μ = 1.0 (KCl))

pH	k_2^{obsd} ($M^{-1}s^{-1}$)[a]
6.28	0.626 ± 0.008
7.23	1.22 ± 0.02
8.26	1.48 ± 0.04

[a] Relative error calculated from the standard deviation of the fit of k_{obsd} vs $[RSH]_{total}$ at the given pH level.

Table 4S : Second Order Rate Constants at Various pH Values for the Reaction of 2-Mercaptomethyl-N-methylimidazole (**7b**) with Distorted Amide **2** (T = 25 °C, μ = 1.0 (KCl))

pH	k_2^{obsd} ($M^{-1}s^{-1}$)[a]
5.39	8.25 ± 0.24
5.99	19.2 ± 0.2
6.29	22.1 ± 0.6
6.85	48.2 ± 1.1
7.92	71.7 ± 2.7
8.35	70.1 ± 2.7
8.60	63.7 ± 2.8
8.95	50.4 ± 0.9
9.74	17.7 ± 0.6
10.32	5.65 ± 0.25

[a] Relative error calculated from the standard deviation of the fit of k_{obsd} vs $[RSH]_{total}$ at the given pH level.

Table 5S : Second Order Rate Constants at Various pH Values for the Reaction of 3-Mercaptopropylamine (**10d**) with Distorted Amide **2** (T = 25 °C, μ = 1.0 (KCl))

pH	k_2^{obsd} ($M^{-1}s^{-1}$)[a]
7.27	0.22 ± 0.05
8.46	2.12 ± 0.02
9.33	8.44 ± 0.30
10.55	14.8 ± 0.3

[a] Relative error calculated from the standard deviation of the fit of k_{obsd} vs $[RSH]_{total}$ at the given pH level.

Table 6S : Second Order Rate Constants at Various pH Values for the Reaction of 3-Mercaptopropylamine (**10d**) with Distorted Amide **2** in D_2O (T = 25 °C, μ = 1.0 (KCl))

pD	k_2^{obsd} ($M^{-1}s^{-1}$)[a]
9.08	3.25 ± 0.13
9.75	9.66 ± 0.08
10.9	14.9 ± 0.2

[a] Relative error calculated from the standard deviation of the fit of k_{obsd} vs $[RSH]_{total}$ at the given pH level.

Table 7S : Second Order Rate Constants[a] at Various Concentrations of N-Methyl-imidazole Buffer for the Reaction of 4-(2-Mercaptoethyl)morpholine (**10c**) with Distorted Amide **2** (pH 7.4, T = 25 °C, μ = 1.0 (KCl))

[Buffer] (M)	k_2^{obsd} ($M^{-1}s^{-1}$)
0.05	3.33 ± 0.17
0.1	4.80 ± 0.24
0.2	8.14 ± 0.41
0.3	13.6 ± 0.7
0.5	19.2 ± 1.0
1.0	37.7 ± 1.9
2.0	82.4 ± 9.4

[a] Calculated from the observed pseudo first-order rate constant, at 1.02 mM thiol concentration. Relative error from buffer concentration, thiol concentration, pH measurement and standard deviation of kinetic fit estimated at about 5 %.

Table 8S : Second Order Rate Constants[a] at Various Concentrations of N-Methyl-imidazole Buffer for the Reaction of 4-(2-Mercaptoethyl)morpholine (**10c**) with Distorted Amide **2** (pH 8.4, T = 25 °C, μ = 1.0 (KCl))

[Buffer] (M)	k_2^{obsd} ($M^{-1}s^{-1}$)
0.1	1.65 ± 0.09
0.2	2.55 ± 0.13
0.5	5.12 ± 0.26
1.0	10.8 ± 0.5

[a] Calculated from the observed pseudo first-order rate constant, at 1.02 mM thiol concentration. Relative error from buffer concentration, thiol concentration, pH measurement and standard deviation of kinetic fit estimated at about 5 %.

Table 9S : Second Order Rate Constants[a] at Various Concentrations of N-Methyl-imidazole Buffer for the Reaction of 4-(2-Mercaptoethyl)morpholine (**10c**) with Distorted Amide **2** in D_2O (pD 7.9, T = 25 °C, μ = 1.0 (KCl))

[Buffer] (M)	k_2^{obsd} ($M^{-1}s^{-1}$)
0.1	5.70 ± 0.46
0.2	10.8 ± 0.9
0.5	24.1 ± 1.9
1.0	48.0 ± 3.8

[a] Calculated from the observed pseudo first-order rate constant, at 1.02 mM thiol concentration. Relative error from buffer concentration, thiol concentration, pH measurement and standard deviation of kinetic fit estimated at about 8 %.

Table 10S : Second-order Rate Constants[a] at Various Concentrations of N-Methylimidazole Buffer for the Reaction of 2-Mercaptomethyl-N-methylimidazole (**7b**) with Distorted Amide **2** (pH 7.3, T = 25 °C, μ = 1.0 (KCl))

[Buffer] (M)	k_2^{obsd} ($M^{-1}s^{-1}$)
0.1	72 ± 7
0.2	76 ± 7
0.5	68 ± 7

[a] Calculated from the observed pseudo first-order rate constant, at 1.03 mM thiol concentration. Relative error from buffer concentration, thiol concentration, pH measurement and standard deviation of kinetic fit estimated at about 10 %.

Table 11S : Second-order Rate Constants[a] at Various Concentrations of N-Methyl-imidazole Buffer for the Reaction of Cysteamine (**10a**) with Distorted Amide **2** (pH 7.8, T = 25 °C, μ = 1.0 (KCl))

[Buffer] (M)	k_2^{obsd} ($M^{-1}s^{-1}$)
0.1	15.7 ± 1.1
0.2	21.4 ± 1.5
0.5	27.9 ± 2.0

[a] Calculated from the observed pseudo first-order rate constant, at 1.01 mM thiol concentration. Relative error from buffer concentration, thiol concentration, pH measurement and standard deviation of kinetic fit estimated at about 7 %.

Derivation of Equation (2):

$$\text{Rate} = \frac{dP}{dt} = k_2 [RS^-] [2]$$

$$Ka^{SH} = \frac{[RS^-] [H^+]}{[RSH]} \; ; \; [RSH] = \frac{[RS^-] [H^+]}{Ka^{SH}}$$

$$[RSH]_{tot} = [RS^-] + [RSH] \; ; \; f_{RS^-} = \frac{[RS^-]}{[RSH]_{tot}} = \frac{[RS^-]}{[RS^-] + [RSH]}$$

$$= \frac{[RS^-]}{\frac{[RS^-] [H^+]}{Ka^{SH}} + [RS^-]} = \frac{Ka^{SH}}{[H^+] + Ka^{SH}}$$

$$\text{Rate} = k_2 (f_{RS^-} [RSH]_{tot}) [2] \; ; \; k_2^{obsd} = \frac{k_2 Ka^{SH}}{[H^+] + Ka^{SH}}$$

Derivation of Equation (3):

$$\text{Rate} = \frac{dP}{dt} = (k_2'\,[ZW] + k_2''\,[ImS^-])\,[pNPA] \quad \text{(Let "ImSH" represent compound 7b.)}$$

$$Ka^{Im} = \frac{[N]\,[H^+]}{[HIm^+SH]} \;;\; Ka^{SH} = \frac{[ImS^-]\,[H^+]}{[N]} \;;\; K_{ZW} = \frac{[ZW]}{[N]} \quad \text{(from Scheme 17)}$$

$$[N] = \frac{Ka^{Im}\,[HIm^+SH]}{[H^+]} \;;\; [ImS^-] = \frac{Ka^{SH}\,[N]}{[H^+]} = \frac{Ka^{SH}\,Ka^{Im}\,[HIm^+SH]}{[H^+]^2}$$

$$[ZW] = K_{ZW}\,[N] = \frac{K_{ZW}\,Ka^{Im}\,[HIm^+SH]}{[H^+]}$$

$$[ImSH]_{tot} = [HIm^+SH] + [N] + [ZW] + [ImS^-]$$

$$f_{ZW} = \frac{[ZW]}{[HIm^+SH] + [N] + [ZW] + [ImS^-]}$$

$$f_{ZW} = \frac{\dfrac{K_{ZW}\,Ka^{Im}\,[HIm^+SH]}{[H^+]}}{[HIm^+SH] + \dfrac{Ka^{Im}\,[HIm^+SH]}{[H^+]} + \dfrac{K_{ZW}\,Ka^{Im}\,[HIm^+SH]}{[H^+]} + \dfrac{Ka^{SH}\,Ka^{Im}\,[HIm^+SH]}{[H^+]^2}}$$

$$= \frac{K_{ZW}\,Ka^{Im}\,[H^+]}{[H^+]^2 + Ka^{Im}\,[H^+] + K_{ZW}\,Ka^{Im}\,[H^+] + Ka^{SH}\,Ka^{Im}}$$

Likewise,

$$f_{ImS^-} = \frac{[ImS^-]}{[HIm^+SH] + [N] + [ZW] + [ImS^-]}$$

$$f_{ImS^-} = \frac{\dfrac{Ka^{SH}\,Ka^{Im}\,[HIm^+SH]}{[H^+]^2}}{[HIm^+SH] + \dfrac{Ka^{Im}\,[HIm^+SH]}{[H^+]} + \dfrac{K_{ZW}\,Ka^{Im}\,[HIm^+SH]}{[H^+]} + \dfrac{Ka^{SH}\,Ka^{Im}\,[HIm^+SH]}{[H^+]^2}}$$

$$= \frac{Ka^{SH}\,Ka^{Im}}{[H^+]^2 + Ka^{Im}\,[H^+] + K_{ZW}\,Ka^{Im}\,[H^+] + Ka^{SH}\,Ka^{Im}}$$

$$\text{Rate} = \frac{dP}{dt} = (k_2'\, f_{ZW} + k_2''\, f_{ImS^-})\,[ImSH]_{tot}[pNPA]\,;$$

$$k_2^{obsd} = \frac{k_2'\,K_{ZW}\,Ka^{Im}\,[H^+] + k_2''\,Ka^{SH}\,Ka^{Im}}{[H^+]^2 + (Ka^{Im} + K_{ZW}\,Ka^{Im})\,[H^+] + Ka^{SH}\,Ka^{Im}}$$

Derivation of Equation (4):

$$\text{Rate} = \frac{dP}{dt} = k_2\,[ZW]\,[2]$$

$[ZW] = f_{ZW}\,[ImSH]_{tot}$, derived as for equation (3).

Rate = $(k_2\, f_{ZW})\,[ImSH]_{tot}\,[2]$;

$$k_2^{obsd} = \frac{k_2\, K_{ZW}\, Ka^{Im}\,[H^+]}{[H^+]^2 + (\,Ka^{Im} + K_{ZW}\, Ka^{Im}\,)\,[H^+] + Ka^{SH}\, Ka^{Im}}$$

APPENDIX 2

Supplementary Material to Chapter 2

Table 12S : Observed Pseudo-First Order Rate Constants vs pH for the Disappearance of **13b** (μ = 1.0 (KCl), T = 25 °C)

pH	$k^{obsd} \times 10^4$ (s^{-1})[a]
6.21[b]	1.94 ± 0.07
7.38[b]	2.15 ± 0.03
9.25[c]	6.13 ± 0.47
10.10[c]	7.15 ± 1.05
10.60[d]	8.50 ± 1.13
10.93[c]	8.14 ± 0.41
12.39[e]	8.25 ± 0.83

[a] Reported values are the averages of duplicate or triplicate runs; relative errors reported are the standard deviations of the average values.

[b] Phosphate buffer.

[c] Carbonate buffer.

[d] Carbonate buffer, D_2O.

[e] Sodium hydroxide.

Table 13S : Observed Pseudo-First Order Rate Constants vs pH for the Disappearance of **17** (μ = 1.0 (KCl), T = 25 °C)

pH	$k^{obsd} \times 10^4$ (s^{-1})[a]
6.21[b]	0.63 ± 0.13
7.38[b]	1.34 ± 0.06
9.25[c]	2.61 ± 0.04
10.10[c]	2.69 ± 1.72

[a] Reported values are the averages of at least duplicate runs; relative errors reported are the standard deviations of the average values.

[b] Phosphate buffer.

[c] Carbonate buffer.

Derivation of Equation (5):

Let TE-NR_2 represent a thiolester with a pendant amino group.

$$\text{Rate} = \frac{dP}{dt} = k_0\,[\text{TE-NR}_2]_{tot} + k_{cat}\,[\text{TE-NR}_2]$$

$$Ka^{NH+} = \frac{[\text{TE-NR}_2]\,[H^+]}{[\text{TE-}^+\text{NHR}_2]}\;;\; [\text{TE-NR}_2] = \frac{[\text{TE-}^+\text{NHR}_2]\,Ka^{NH+}}{[H^+]}$$

$$[\text{TE-NR}_2]_{tot} = [\text{TE-NR}_2] + [\text{TE-}^+\text{NHR}_2]$$

$$f_{\text{TE-NR2}} = \frac{[\text{TE-NR}_2]}{[\text{TE-NR}_2]_{tot}} = \frac{[\text{TE-NR}_2]}{[\text{TE-NR}_2] + [\text{TE-}^+\text{NHR}_2]}$$

$$= \frac{\dfrac{[\text{TE-}^+\text{NHR}_2]\,Ka^{NH+}}{[H^+]}}{\dfrac{[\text{TE-}^+\text{NHR}_2]\,Ka^{NH+}}{[H^+]} + [\text{TE-}^+\text{NHR}_2]} = \frac{1}{1 + \dfrac{[H^+]}{Ka^{NH+}}}$$

$$\text{Rate} = k_0\,[\text{TE-NR}_2]_{tot} + k_{cat}\,f_{\text{TE-NR2}}\,[\text{TE-NR}_2]_{tot}$$

$$= (\,k_0 + k_{cat}\,f_{\text{TE-NR2}}\,)\,[\text{TE-NR}_2]_{tot}$$

$$k^{obsd} = k_0 + \frac{k_{cat}}{1 + \dfrac{[H^+]}{Ka^{NH+}}}$$

Table 14S : Observed Pseudo-First Order Rate Constants vs Equivalents of Added Dimethylamioethanethiol (**10b**) for the Disappearance of **13b**[a] (pH 10.10,[b] μ = 1.0 (KCl), T = 25 °C)

Equivalents of **10b**	$k^{obsd} \times 10^4$ (s^{-1})[c]
0	7.6 ± 0.8
5	5.3 ± 0.5
11	5.0 ± 0.5
23	3.8 ± 0.4

[a] **[13b]** = 4.1×10^{-4} M.

[b] Carbonate buffer.

[c] Relative error estimated at 10%.

APPENDIX 3

Supplementary Material to Chapter 3

Table 15S : Observed Pseudo-First Order Rate Constants for the Reaction of **7b** with 100 mM DMF (pD 8.0, T = 98 °C, μ = 1.0 (KCl))

[**7b**] (mM)	$k^{obsd} \times 10^6$ (s^{-1})[a]
0	0.030 ± 0.003
20	0.50 ± 0.05
50	1.13 ± 0.11
100	1.80 ± 0.18
150	2.61 ± 0.26

[a] Relative error in the rate constants is estimated at 10%.

Table 16S : Observed Pseudo-First Order Rate Constants for the Reaction of **7b** with 100 mM Formamide (pD 7.8, T = 98 °C, μ = 1.0 (KCl))

[**7b**] (mM)	$k^{obsd} \times 10^6$ (s^{-1})[a]
0	0.36 ± 0.04
50	6.17 ± 0.36
100	12.2 ± 0.6
150	19.5 ± 1.8

[a] Error shown is standard deviation from non-linear least squares fit of the data.

Table 17S : Observed Pseudo-First Order Rate Constants for the Reaction of **7b** with 200 mM N-Formylmorpholine (pD 8.0, T = 98 °C, μ = 1.0 (KCl))

[**7b**] (mM)	$k^{obsd} \times 10^6$ (s^{-1})[a]
0	0.030 ± 0.001
100	1.13 ± 0.08
150	1.63 ± 0.13

[a] Error shown is standard deviation of linear least squares analysis of [NFM] vs time data. Rate constants were calculated by dividing the initial slopes by the initial concentration of amide (200 mM).

Table 18S : Observed Pseudo-First Order Rate Constants for the Formation of NFM by the Reaction of **7b** with Formic Acid and Morpholine (T = 98 °C, μ = 1.0 (KCl))

[**7b**] (mM)	[HCOOH] (mM)	$k^{obsd} \times 10^7$ (s^{-1})[a]
0	200	0.95 ± 0.04[b]
100	200	1.21 ± 0.06[b]
100	400	2.94 ± 0.24[c]

[a] Pseudo-first order rate constants were calculated by dividing the initial slope of [NFM] vs time by the initial concentration of formic acid (initial rates method). Relative error shown is the standard deviation of the linear least squares fit of the initial rate data.

[b] pD 7.6, 200 mM morpholine.

[c] pD 7.7, 400 mM morpholine. Note that the observed pseudo-first order rate constant approximately doubled on doubling the concentrations of both formic acid and morpholine. (The fact that the observed rate did **not** *quadruple*, and that no build-up of thiolester was observed under any conditions indicates that only formic acid is involved, with the catalyst, in the rate-determining step.)

Derivation of Equation (6):

Let "TE" represent the intermediate thiolester.

If [TE] is in a steady state, then from **Scheme 26**,

$$\frac{d[TE]}{dt} = k_{dehyd}[acid][cat] - k_{hyd}[TE] - k_{form}[TE][amine] = 0$$

$$[TE] = \frac{k_{dehyd}[acid][cat]}{k_{form}[amine] + k_{hyd}}$$

Let the rate of amide production be given by V_f.

$$V_f = k_{form}[TE][amine] = \frac{k_{dehyd}\,k_{form}[acid][cat][amine]}{k_{form}[amine] + k_{hyd}}$$

Derivation of Equation (8):

If [TE] is in a steady state in the reverse direction of **Scheme 26**,

$$\frac{d[TE]}{dt} = k_{cat}[amide][cat] - k_{hyd}[TE] - k_{form}[TE][amine] = 0$$

$$[TE] = \frac{k_{cat}[amide][cat]}{k_{form}[amine] + k_{hyd}}$$

Let the rate of acid production be given by V_r.

$$V_r = k_{hyd}[TE] = \frac{k_{hyd}\,k_{cat}[amide][cat]}{k_{form}[amine] + k_{hyd}}$$

Derivation of Equation (10):

$$Ka^{FA} = \frac{[HCOO^-]\,[H_3O^+]}{[HCOOH]} \qquad [HCOOH] = \frac{[HCOO^-]\,[H_3O^+]}{Ka^{FA}}$$

$$Ka^{NH+} = \frac{[morph]\,[H_3O^+]}{[morph\text{-}H^+]} \qquad [morph] = \frac{Ka^{NH+}\,[morph\text{-}H^+]}{[H_3O^+]}$$

$$[HCOOH] = f_{FA}\,[HCOOH]_{tot} \qquad f_{FA} = \frac{[HCOOH]}{[HCOOH] + [HCOO^-]}$$

$$f_{FA} = \frac{\dfrac{[HCOO^-]\,[H_3O^+]}{Ka^{FA}}}{\dfrac{[HCOO^-]\,[H_3O^+]}{Ka^{FA}} + [HCOO^-]} = \frac{[H_3O^+]}{[H_3O^+] + Ka^{FA}}$$

$$[morph] = f_{morph}\,[morph]_{tot} \qquad f_{morph} = \frac{[morph]}{[morph] + [morph\text{-}H^+]}$$

$$f_{morph} = \frac{\dfrac{Ka^{NH+}\,[morph\text{-}H^+]}{[H_3O^+]}}{\dfrac{Ka^{NH+}\,[morph\text{-}H^+]}{[H_3O^+]} + [morph\text{-}H^+]} = \frac{Ka^{NH+}}{Ka^{NH+} + [H_3O^+]}$$

$$K_{eq} = \frac{[NFM]}{[HCOOH]\,[morph]}$$

$$= \frac{[NFM]}{\left(\dfrac{[H_3O^+]}{[H_3O^+] + Ka^{FA}}\right)[HCOOH]_{tot}\left(\dfrac{Ka^{NH+}}{Ka^{NH+} + [H_3O^+]}\right)[morph]_{tot}}$$

$$= \frac{K'_{eq}}{\text{"F"}} \;;\; K'_{eq} = \text{"F"}\,K_{eq}$$

From reference 86, $K_{eq} = 2.24 \times 10^5\ M^{-1}$, $pKa^{FA} = 3.66$, $pKa^{NH+} = 8.87$.

In D_2O, $pKa^{FA} = 4.06$, $pKa^{NH+} = 9.27$

(see reference 71).

At pD 7.6 (under conditions used herein), "F" $= 6.00 \times 10^{-6}$, and

$$K'_{eq} = \text{"F"}\ K_{eq} = (6.00 \times 10^{-6})\ (2.24 \times 10^5\ M^{-1}) = 1.35\ M^{-1}$$