Attack of Zwitterionic Ammonium Thiolates on a Distorted Anilide as a Model for the Acylation of Papain by Amides. A Simple Demonstration of a Bell-Shaped pH/Rate Profile

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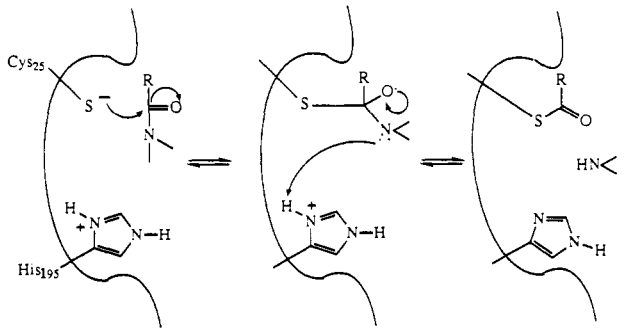
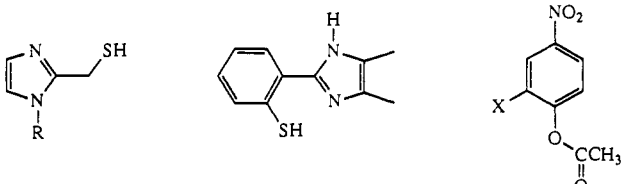
Abstract: A distorted anilide (**4**) has been shown to be remarkably susceptible to attack by thiolates containing pendant N-H$^+$ groups. Thiolates alone are not reactive nor are amines or ammonium ions. Attack of the zwitterionic form produces a thiol ester, which is isolable in the case of *N,N*-dimethylcysteamine. If the pendant amine is primary, as in the case of cysteamine, the isolable product is the amide formed by intramolecular S to N acyl transfer. The kinetics of the reaction of 2-(mercaptomethyl)-*N*-methylimidazole (**1b**) on **4** show a bell-shaped pH vs log k_2 profile that results from attack of the zwitterionic form on the amide. The role of the pendant ammonium group is to serve as a general acid trapping agent of the unstable tetrahedral intermediate formed from H-N-$^+$⋯S$^-$ attack on the amide C=O.

The cysteine proteases comprise a large class of enzymes from plant, animal, and bacterial sources,[1] the active sites of which contain an essential cysteine SH and histidine imidazole unit.[2] The catalytic pathway for hydrolysis of both ester and amide substrates involves formation of a cysteine *S*-acyl enzyme.[1,3] The acylation process follows bell-shaped pH/rate profiles for typical amide substrates and depends upon two active residues having pK_a values of ~3.9–4.3 and 8.2–8.5.[4] These ionizations have been attributed to the imidazolium and SH residues[5,6] although there was some early ambiguity about which group had the lowest pK_a. It is now generally accepted[1h,6] that in papain, Cys-25 has the unusually low pK_a of 3–4, while the histidine-195 imidazolium ion has the pK_a of ~8.5. Thus, it is proposed[7a] that at physiological pH, 90% of the papain exists in a form wherein the active site consists of a kinetically competent[6,7] imidazolium thiolate zwitterionic pair. A highly stylized representation of the pathway for acyl transfer from amides mediated by papain is presented in Scheme I.

Scheme I



A number of small molecules containing both imidazole and SH have been investigated as models for the acylation step of papain with esters,[8] but, for the most part, these have shown little or no cooperative effect of the imidazole in S-acylation such as is believed to occur in the cysteine proteases. However, earlier reports[9] from our laboratories have shown that imidazole thiols **1** and **2** react rapidly as their zwitterionic forms with the activated esters *p*-nitrophenylacetate (pNPA, **3a**) and 2,4-dinitrophenyl-



1a R=H
b R=CH$_3$

2

3a X=H
b X=NO$_2$

acetate (DNPA, **3b**), respectively. In those cases there is no sign of a bell-shaped pH rate profile, and, throughout the pH range from 5–10, the rate limiting step involves nucleophilic thiolate attack on the ester. The imidazolium unit plays the passive role of electrostatically reducing the SH pK_a so that large concentrations of nucleophilic thiolate are present at neutral pH.

Few studies have been reported where amino thiols have been used to facilitate cleavage of the amide bond.[10] The reported

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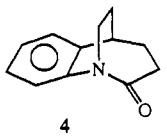
studies involved intramolecular thiolactonization of unactivated amides[10a,b,d] and bimolecular attack of 2-mercaptoethanol on the activated amide, *N*-acetylimidazole,[10c] but it is surprising that bimolecular attack of thiols on amides has not been studied extensively as a model for the chemistry believed to be operative in the cysteine proteases. Undoubtedly, this is due to the resistance of the unactivated N–C(O) unit toward nucleophilic attack which precludes such studies under all but the most vigorous conditions. We have shown earlier that a distorted anilide (**4**) is remarkably susceptible toward attack by bifunctional nucleophiles such as β-amino alcohols[11] or certain dicarboxylic acids[12] in ways that are reminiscent of hydrolysis reactions promoted by the serine proteases and aspartate proteinases. Herein we describe the reactions of **4** with certain amino thiols (**1b**, **5**) as a simple model system for the acylation step of the active site SH in cysteine proteases by amides.[13] These studies reveal (1) that the zwitterionic forms $>N^+$-H⋯S^- are responsible for nucleophilic attack, (2) the origin of a bell-shaped pH/rate profile for reaction of **1b** with **4**, and (3) significant differences in the catalytic requirements for the S-acylation of aminothiols by the amide, **4**, and the activated ester, pNPA.



4

$HSCH_2CH_2X$ $RSCH_2X$

5a X=NH_2 c X=N⟨ ⟩O 6a R=H; X=CH_3
b X=$N(CH_3)_2$ d X=CH_2NH_2 b R=H; X=CO_2Et
c R=H; X=CH_2CN
d R=Et; X=CH_2NH_2

Experimental Section

(a) General. Routine IR and ^1H NMR and mass spectra were recorded on a Nicolet 7199-FTIR spectrophotometer, a Bruker WP-80 (80 MHz) spectrometer, and an AEI MS-50 mass spectrometer, respectively. High field NMR spectra (^1H and ^{13}C) were recorded on a Bruker AM-300 (300 MHz) machine.

(b) Materials. 2-Mercaptoethylamine (cysteamine, **5a**), *N,N*-(dimethylamino)ethanethiol (dimethylcysteamine, **5b**), ethyl mercaptoacetate (**6b**), ethanethiol (**6a**), and (2-(ethylthio)ethyl)amine (**6d**) were commercially available (Aldrich) and were used as supplied if solid or after distillation if liquid. Buffers (MES, morpholinoethanesulfonic acid; MOPS, morpholinopropanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid; TAPS, ((tris(hydroxymethyl)-methyl)amino)propanesulfonic acid; CHES, (cyclohexylamino)ethanesulfonic acid; and CAPS, (cyclohexylamino)propanesulfonic acid) were reagent grade (Sigma) and used as supplied. Acetonitrile was dried over 3Å molecular sieves, then distilled, and stored under Ar. Amide **4** (2,3,4,5-tetrahydro-2-oxo-1,5-ethanobenzazepine) was prepared as reported.[14] 2-(Mercaptomethyl)-*N*-methylimidazole (**1b**) was prepared as reported.[9b] 4-(2-Mercaptomethyl)morpholine (**5c**) and 3-mercaptopropionitrile (**6c**) were prepared from the corresponding commercially available alkyl chlorides by displacement with thiourea according to methods analogous to a published procedure.[15]

4-(2-Mercaptoethyl)morpholine·HCl (5c·HCl). This was prepared from 2-(4-morpholino)ethyl carbamimidothioate dihydrochloride in 80% yield: mp 156–156.5 °C; ^1H NMR (D_2O) δ 4.60 (s, >2 H), 3.98 (t, 4 H), 3.40 (t, 6 H), 2.89 (t, 2 H); FTIR (Br, cm^{-1}) 3400 (br), 3000–2900 (s), 2800–2000 (s, br), 1460. Anal. Calcd for $C_6H_{13}NOS·HCl$: C, 39.23; H, 7.63; N, 7.63; S, 17.45. Found: C, 39.21; H, 7.60; N, 7.43; S, 17.36.

3-Mercaptopropionitrile·HCl (6c·HCl). This was prepared from 3-isothioureidopropionitrile in 84% yield: bp 59–60 °C (4 Torr); ^1H NMR ($CDCl_3$) δ 2.72 (m, 4 H), 1.82 (t, 1 H); FTIR (film) 2947, 2566, 2250, 1418, 1291 cm1-1. Anal. Calcd for $C_3H_5NS·HCl$: C, 41.35; H, 5.78; N, 16.07. Found: C, 40.81; N, 5.62; N, 15.71.

(3-Mercaptopropyl)amine·HCl (5d·HCl). This was prepared by $Na/NH_3(l)$ reduction of the corresponding *S*-((benzylthio)propyl)amine according to the method already published:[9b] yield 50%; mp 68 °C [lit.[16] 69 °C]; ^1H NMR (D_2O) δ 4.64 (s, >4 H), 3.25 (t, 2 H), 3.15 (t, 2 H), 2.65 (t, 2 H), 2.00 (m, 2 H); FTIR (KBr, cm^{-1}) 3400, 2980, 2000, 1600, 1400, 1260; mass spectrum, *m/z* (rel intensity) 91 (M^+ – HCl, 100), 74 (53.1), 58 (49.6).

(c) Kinetics. Buffers (50 mM, μ = 1.0 (KCl)) were used to control the pH: MES (pH 5.4–6.3); MOPS (pH 6.9–7.9); EPPS (pH 8.0–8.3); TAPS (pH 8.4–8.7); CHES (pH 8.6–9.7); CAPS (pH 10.3–11.0). Distilled water was further purified and deoxygenated with an Osmonics Aries water purification system. Buffers were prepared and stored under Ar using the above deoxygenated water.

Kinetics of the ring opening of **4** by excess **1b**, **5a–d**, ethanethiol, ethyl mercaptoacetate, and 3-mercaptopropionitrile were followed at 25.0 ± 0.1 °C by observing the rate of production of the substituted aniline chromophore at 291 nm using a Cary 210 UV–vis spectrophotometer interfaced to a microcomputer as previously described.[17] Deoxygenated aqueous buffers (3.0 mL) were transferred by syringe to an Ar-flushed septum-sealed 1.0-cm quartz cuvette. Varying concentrations of the thiols were introduced by injection of 50–200 μL aliquots of deoxygenated buffer solutions containing 60–65 mM thiol. Runs were initiated (after thermal equilibration of the cell in the spectrophotometer cell holder for ~10 min) by injection of 10–15 μL of a 20–25 mM stock solution of **4** in CH_3CN into the cell. To ensure pseudo-first-order conditions, thiol was kept to a 10–40-fold excess over **4**: no evidence for the build up of tetrahedral intermediates such as biphasic or saturation kinetics was observed in any case. The pH of the solutions was measured after the kinetic runs to ensure there were no changes (Radiometer TTT2 titrator equipped with a GK2321C combination electrode standardized by Fisher Certified pH 4.00, 7.00, and 10.00 buffers). Reactions displayed excellent first-order kinetics to up to 5 half-times. Rate constants (k_{obsd}) were obtained by fitting the abs vs time data to a standard exponential model. Runs with each [thiol] were performed in duplicate or triplicate. Second-order rate constants for the reaction of thiol with **4** (k_2^{obsd}) were obtained from the slopes of the plots of k_{obsd} vs $[thiol]_{total}$ (4–6 concentrations). For the amino thiols **5a–d**, plots of k_2^{obsd} vs pH

Table I. Kinetic Second-Order Rate Constants (k_2) and pK_a^{SH} Values for the Reactions of Thiols with **4** (T = 25.0 ± 0.1 °C, μ = 1.0 KCl)

thiol	k_2[a] (M^{-1} s^{-1})	kinetic pK_a^{SH}[b]	lit. pK_a^{SH}	lit. $pK_a^{NH^+}$
5a	99.2 (100)[d,e]	8.37	8.35[c] (8.74)[d,f]	10.86[c]
5b	30.5	7.73	7.74[g]	10.89[g]
5c	1.47 (1.74)[d,i]	6.44	6.57[h] (6.78)[d,j]	9.55[h]
5d	15.5 (15.7)[d,l]	9.23 (9.59)[d,l]	9.26[k] (9.60)[d,j]	10.84[k]
6a	<0.05[m]		10.55[n]	
6b	0.36		8.03[g]	
6c	<0.02[m]		8.74[g]	
6d	nil			

[a] Relative standard error (from standard deviation of fits of k_2^{obsd} vs $[H_3O^+]$) is less than 5%. [b] Standard error is less than 0.05 pK units as determined from fits of k_2^{obsd} vs $[H_3O^+]$. [c] Benesch, R. E.; Benesch, R. *J. Am. Chem. Soc.* **1955**, *77*, 5877. [d] In D_2O. [e] k_2 calculated from k_2^{obsd} determined at pD = 9.08 and pK_a^{SD} (D_2O) of 8.74. [f] Creighton, D. J.; Schamp, D. J. *FEBS Lett.* **1980**, *110*, 313. [g] Kostyukovskii, Ya. L.; Bruk, Yu. A.; Pavlova, L. V.; Slavachevskaya, N. M.; Kokushkina, A. V.; Mirkin, B. S.; Belen'kaya, I. A. *Zh. Obshch. Khim.* **1972**, *42*, 662. [h] Bagiyan, G. A.; Koroleva, I. K.; Sonoka, N. V. *Zh. Neorgan. Khim.* **1977**, *22*, 3078. [i] k_2 calculated from k_2^{obsd} determined at pD = 8.85 and pK_a^{SD} (D_2O) of 6.78. [j] Calculated as ΔpK_a (H → D) = 0.26 ± 0.012 pK_a^{SH}. Jencks, W. P.; Salvesen, K. *J. Am. Chem. Soc.* **1971**, *93*, 4433. [k] Bagiyan, G. A.; Koroleva, I. K.; Grachev, S. A.; Soroka, N. Y. *Akad. Nauk SSSR Bulletin. Div. Chem. Sci.* **1976**, *25*, 996 gives pK_a^{SH} values of 9.39 (20 °C) and 9.14 (30 °C): value at 25 °C taken as the average. [l] Computed from k_2^{obsd} values at pD 9.08, 9.75, 10.9 and fixed pK_a^{SD} of 9.60. [m] An upper value estimated from detection limit due to slowness of reaction in the presence of the thiol relative to blank reaction. See Experimental Section. [n] Nenasheva, T. N.; Salinkova, G. A. *Zh. Org. Khim.* **1979**, *15*, 835.

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showed evidence of a plateau above pK_a^{SH}. For thiols not containing an amine, the k_2^{obsd} was obtained by determining k_{obsd} at the pK_a of the thiol and then dividing this value by $[RS^-]$. In some cases (**6a,c**) no detectable rate acceleration was observed in the presence of thiol so the k_2^{obsd} values are given as upper limits in Table I.

(d) D_2O Studies. The buffers and stock solutions were prepared under Ar in the way described above, using D_2O which had been purged of O_2 by passing a stream of Ar through it for several hours. pD was determined by adding 0.4 units to the pH meter reading.[18]

(e) Product Studies. Large scale reactions of **4** and cysteamine (**5a**) or dimethylcysteamine (**5b**) were determined under standard kinetic conditions for $\sim 10t_{1/2}$, and the reaction mixtures were extracted and worked up as below to identify the final product.

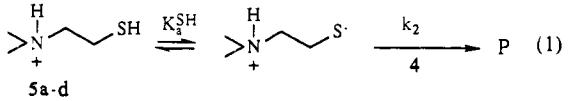
Into 25 mL of 1 M KCl solution was placed ~3 mL of a 62 mM cysteamine solution (in degassed H_2O) and 0.83 mL of a 22 mM solution of **4** in CH_3CN. The pH was kept constant at ~8.6 for 30 min by the addition of small amounts of dilute NaOH, after which the mixture was extracted with 3 × 20 mL aliquots of distilled CH_2Cl_2. The organic extracts were combined, dried ($MgSO_4$), and then stripped of solvent. The residue was shown by ^1H and ^{13}C NMR to contain a predominant species having peak positions identical to the ring opened *amide* product (**9a**) synthesized independently by an unambiguous route (see supplementary material).

For the reaction of amide **4** with dimethylcysteamine (**5b**) the above procedure was followed, and the product was characterized by ^1H and ^{13}C NMR and IR spectroscopy as the corresponding thiol ester (**8b**) (see supplementary material).

The product of the reaction of **4** with **1b** was determined in an NMR experiment wherein the spectrum of a solution consisting of 17.6 mg of **3b**, 100 μL of 1.06 N KOD, 600 μL of CD_3CN and 20.2 mg of **4** was monitored periodically. The thiol ester was formed within ~10 min: 300 MHz ^{13}C NMR δ 199.5, 145.8, 144.5, 129.9, 127.9, 126.5, 124.8, 123.3, 117.1, 115.2, 41.9, 38.4, 35.6, 32.7, 26.5, 24.7 ppm. The volatiles were removed and a FTIR spectrum of a CH_2Cl_2 cast was taken: 3360, 3280, 2920, 1689, 1648, 1605, 1581, 1491 cm^{-1}. By the characteristic ^{13}C resonance at δ 199.5, the product was identified as the corresponding thiol ester.[19]

Results

(a) Kinetics. The kinetics of the reactions of the various thiols with **4** were followed at 25 °C and H = 1.0 (KCl), under pseudo-first-order conditions of excess thiol to give k_{obsd}. The observed second-order rate constants (k_2^{obsd}) were evaluated from the slopes of the plots of k_{obsd} vs $[thiol]_{total}$ at several pH values. For amino thiols **5a–d**, the k_2^{obsd} values plateau above the pK_a value for the thiol which indicates that the neutral or, more probably, the kinetically equivalent zwitterionic form is the active one. Overall, the data support the process in eq 1 for which can be derived the kinetic expression given in eq 2. Given in Table I are the various



(1)

$$k_2^{obsd} = k_2K_a^{SH}/(K_a^{SH} + [H_3O^+]) \quad (2)$$

maximal second-order rate (k_2) and kinetic acidity (pK_a^{SH}) constants derived for **5a–d** on the basis of the fit of the k_2^{obsd} vs $[H_3O^+]$ data to eq 2 by nonlinear least squares techniques. Also in the table are the thermodynamic macroscopic pK_a values for the SH and N^+H groups from literature sources.

Thiols **6a–c** are far less reactive toward **4** than the amino thiols: this suggests a pendant amino (ammonium) functionality is necessary for activity. Also, the blocked S derivative of cysteamine, namely (2-(ethylthio)ethyl)amine (**6d**) does not react with **4** at all in the studied pH range of 7–9. Thus, the ammonium (amine) functionality is insufficient alone to generate activity. The k_2^{obsd} values for thiols **6a–c** were evaluated at the pH equivalent to the pK_a^{SH}: twice the k_2^{obsd} are the maximal k_2 values given in Table I. In fact, **6a** and **6c** exhibited no activity toward **4** under the conditions investigated so that the values given in the table are upper limits.

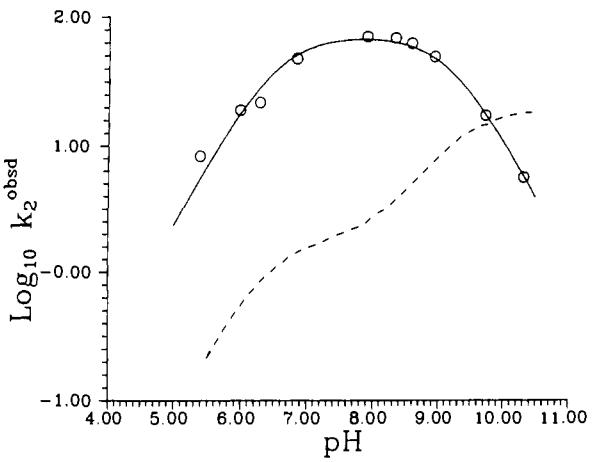
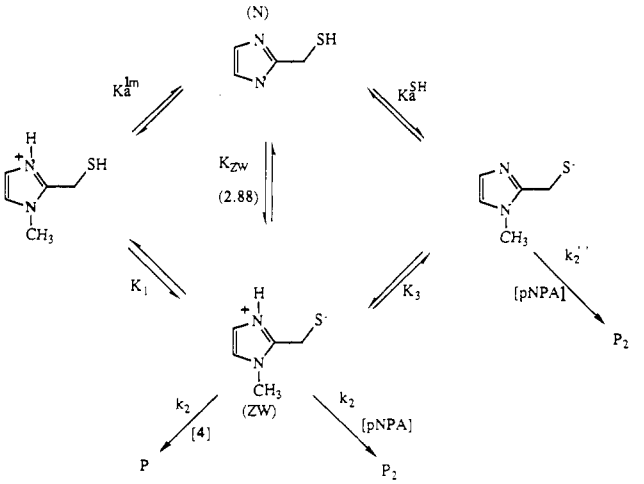


Figure 1. Plots of the log k_2^{obsd} vs pH profile for the reaction of **1b** with **4**; T = 25 °C, μ = 1.0 (KCl). Line through the data computed on the basis of NLLSQ fit to eq 3. Dashed line represents reaction of **1b** with *p*-nitrophenyl acetate: T = 37 °C, μ = 0.3 (KCl), from ref 9.

Scheme II



The pH vs log k_2^{obsd} profile for the reaction of **1b** with **4** is presented in Figure 1, along with a previously determined[9] profile for the reaction of **1b** with *p*-nitrophenyl acetate (pNPA). The striking aspect is the bell-shaped appearance of the former profile which indicates that the activity toward the amide depends upon the state of ionization of both the SH and Im-H^+ residues. The profile is clearly different from that presented for pNPA which suggests the mechanisms operative for the two cases are different. Previously,[9] we have shown that **1b** exists in solution as the four microscopic pH dependent forms given in Scheme II and that the two thiolate forms are reactive toward pNPA. In that scheme are defined the microscopic pK_a values[20] and the value $K_{ZW} = K_1/K_a^{Im} = [ZW]/[N] = 2.88$ which indicates that at neutrality ~70% of the formally neutral species in solution are zwitterionic.

From the various species and constants shown in Scheme II can be derived kinetic expression 3 in which it is assumed that only the zwitterionic form of **1b** reacts with **4**. Nonlinear least

$$k_2^{obsd} = \frac{(k_2K_a^{Im}K_{ZW})[H_3O^+]}{[H_3O^+]^2 + (K_a^{Im} + K_a^{Im}K_{ZW})[H_3O^+] + K_a^{Im}K_a^{SH}} \quad (3)$$

squares (NLLSQ) fitting of the k_2^{obsd} vs $[H_3O^+]$ data for attack of **1b** on **4** to eq 3[21] yields the kinetic constants given in Table

(18) Fife, T. H.; Bruice, T. C. *J. Chem. Phys.* **1961**, *65*, 1079.

(19) Scott, A. I. *Pure Appl. Chem.* **1986**, *58*, 753 reports that thiol esters generally exhibit $^{13}C{=}O$ resonances in the range of 195–200 ppm ~20 ppm downfield from their corresponding carboxylic acids. In the case of the authentic amino acid of **4**, the $^{13}C{=}O$ peak is at 176.6 ppm (CD_3CN containing 13 vol % H_2O).

(20) The pK_a values quoted in Scheme I are the correct ones: those reported in ref 9, Table IV for 2-(thiomethyl)imidazole, and *N*-methyl-2-(thiomethyl)imidazole should be interchanged.

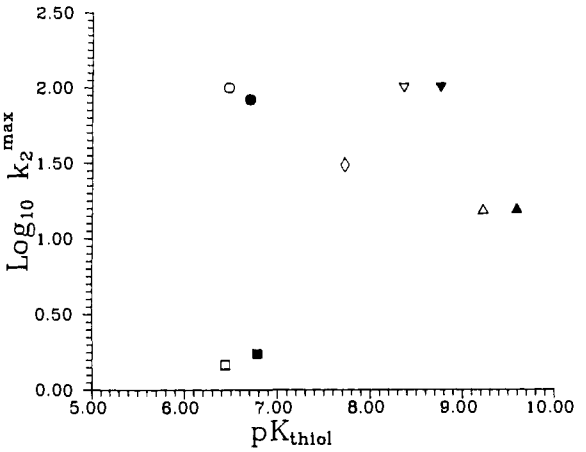


Figure 2. A plot of the maximal second-order rate constant (k_2) for attack of amino thiols **1b** and **5a–d** on **4** vs the pK_a^{SH} values in H_2O (open symbols) and D_2O (closed symbols): T = 25 °C, μ = 1.0 (KCl) (**1b** (○, ●), **5a** (▽, ▼), **5b** (◇), **5c** (□, ■), **5d** (△, ▲)).

Table II. Kinetic Second-Order Rate Constants (k_2) and pK_a Values for Attack of **1b** on **4** (T = 25 °C, μ = 1.0 (KCl))[a]

parameter	kinetic value	lit. value[b]	parameter	kinetic value	lit. value[b]
k_2 (M^{-1} s^{-1})	99 ± 11		pK_a^{SH}	8.66 ± 0.10	8.67
	(83 ± 7)[c]		pK_a^1	6.48[d]	6.37
pK_a^{Im}	7.07 ± 0.10	6.96	pK_a^2	9.25[d]	9.26

[a] Constants defined as in Scheme I. [b] From refs 9 and 20. [c] Determined in D_2O at pD = 7.95, the crest of the pD vs log k_2^{obsd} profile. [d] Macroscopic pK_a values computed from K_{ZW} = 2.88 and equations given in ref 22.

II from which is calculated the line through the data in Figure 2. The fitting requires setting K_{ZW} to the value (2.88) determined previously by titration and UV–visible spectrophotometry[20,9] and generates the maximal rate constant, k_2, and the two kinetic microscopic dissociation constants, pK_a^{Im} and pK_a^{SH}. From these can be determined all of the microscopic pK_a values as well as the macroscopic ones[22] which are presented in Table II along with the literature values.[20]

(b) D_2O Studies. Solvent kinetic isotope effects (skie) for the reactions of **1b** and **5a,c,d** with **4** were determined, and the maximal k_2 values are presented in Tables I and II. In the case of **5d**, the complete pD vs log k_2^{obsd} profile was determined and fit to eq 2 by NLLSQ techniques to yield both k_2 and K_a^{SD}. For **5a,c**, the pK_a^{SD} values were computed according to the equation presented by Jencks and Salvesen:[23] this value and k_2^{obsd} at the pD equivalent to the pK_a^{SD} of the amino thiol were used to calculate the k_2 presented in Table I. For **1b**, the $k_2(D_2O)$ was determined at the crest of the bell-shaped profile of pL vs k_2^{obsd}. $(pK_1)_D$ (Scheme I) was calculated by the method of Jencks and Salvesen, while $(pK_a^{Im})_D$ was assumed to increase by 0.4 units from the value in H_2O, as a consequence of the increased acidity of D_3O^+ relative to H_3O^+.[24] From these values the percentage of ZW can be estimated which, when normalized to 100%, allows calculation of the $(k_2)_D$ given in Table II.

Discussion

For the amino thiols **5a–d** and comparison species **6**, the available evidence for the reaction with **4** supports the process given in Scheme III. The facts that require reconciliation are as follows.

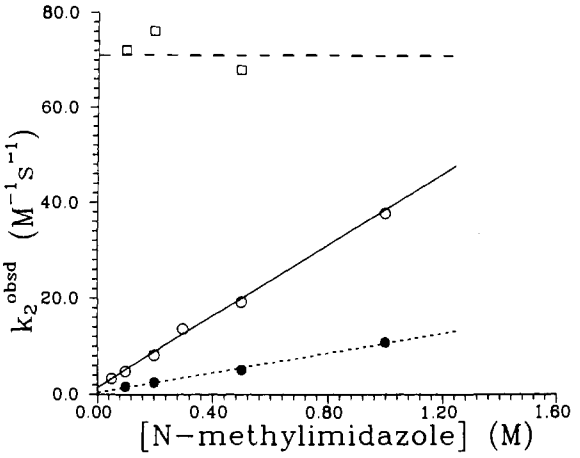


Figure 3. Plots of log k_2^{obsd} for attack of **1b** (□, pH = 7.3) and **5c** (○, pH 7.4; ●, pH 8.4) on **4** as a function of added *N*-methylimidazole: T = 25 °C, μ = 1.0 (KCl).

1. The amino thiols are active above the pK_a^{SH}. This implies that the zwitterion, or far less likely, the neutral amino thiol is the kinetically competent form. Thiols **6a–c** which contain no pendant amino group show little or no activity. Also the S-blocked derivative $CH_3CH_2SCH_2CH_2NH_2$ (**6d**) shows no activity indicating that the presence of an amino group alone is insufficient to bring about reaction with **4**.

2. The product of the reaction of **4** with the N-blocked derivative **5b** is thio ester **8b**. In the case of cysteamine (**5a**, R = H) the isolated product is the corresponding amide, **9a**. Given that the thiolate is required for activity (compare **5a** and **6d**), amide **9a** is likely formed by an intramolecular S → N acyl transfer from **8a**. Reaction of **4** with **1b** in CH_3CN generates the corresponding thiol ester.

Scheme III predicts that the role of the ammonium group is to trap the initially produced tetrahedral intermediate (**7**) by intramolecular proton transfer to produce 7^N or $7^{\pm}$, the latter being poised for breakdown to the observed thiol ester **8**. Such intramolecular trapping drives the reaction forward by preventing the expulsion of thiolate to reform **4** and ammonium thiolate. Probably, thiolate anion alone can attack **4**, but the reaction is reversible and unproductive unless the unstable tetrahedral intermediate can be trapped. In the scheme, we cannot tell whether the intramolecular trapping protonation proceeds to N (to yield $7^{\pm}$) or O (to yield 7^N). Protonation of the latter to yield 7^N would be thermodynamically preferred,[25] but at some point the proton must be transferred to N to give $7^{\pm}$ which is almost certainly the form required for breakdown.

Several pieces of evidence support the above. First, Jencks and co-workers[26] have shown that the microscopic reverse reaction, intramolecular aminolysis of a thiol ester, is subject to general base catalysis. This has been proposed to result from rate-limiting deprotonation of the ammonium ion in the tetrahedral intermediate analogous to $7^{\pm}$, causing expulsion of the thiolate anion to be favored. Second, although the alkyl thiols **6a–c** showed little activity alone toward **4**, the thiolysis reaction is subject to catalysis by such buffering agents as CAPS, CHES, and EPPS.[27] Thiolysis of **4** by **5c** is also subject to buffer catalysis by *N*-methylimidazole, but we have been unable to detect buffer catalysis of the reaction

(21) The actual equation fit was

$$k_2^{obsd} = (k_2K_{ZW})[H^+]/\left(\frac{[H^+]^2}{K_a^{Im}} + (1 + K_{ZW})[H^+] + K_a^{SH}\right)$$

(22) The macroscopic K_a values are defined as

$$K_a^1 = K_a^{Im} + K_1 \text{ and } 1/K_a^2 = 1/K_a^{SH} + 1/K_3.$$

(23) Jencks, W. P.; Salvesen, K. *J. Am. Chem. Soc.* **1971**, *93*, 4433.
(24) Alvarez, F. J.; Schowen, R. L. *Isotopes in Organic Chemistry, Vol. 7*. In *Secondary and Solvent Isotope Effects*, Buncel, E., Lee, C. C., Eds.; Elsevier: Amsterdam, 1987; pp 1–60.
(25) Estimated on the basis of values given for N–H^+ ionization of the zwitterionic form of the $HOCH_3$ addition product of dehydroquinolone: Kirby, A. J.; Mujahid, T. G.; Camilleri, P. *J. Chem. Soc., Perkin Trans. II* **1979**, 1610. Fox, J. P.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 1436.
(26) (a) Barnett, R. E.; Jencks, W. P. *J. Am. Chem. Soc.* **1969**, *91*, 2358. (b) Barnett and Jencks *J. Am. Chem. Soc.* **1968**, *90*, 4199.
(27) Keillor, J. W.; Brown, R. S. unpublished observations CAPS = 3-(*N*-cyclohexylamino)propanesulfonic acid; CHES = 2-(*N*-cyclohexylamino)ethanesulfonic acid; EPPS = *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid.

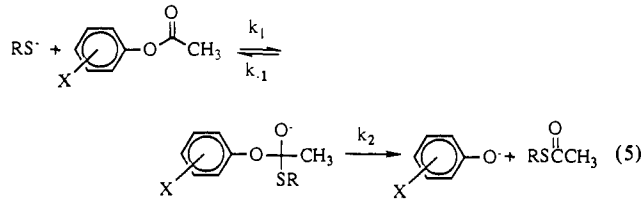
of **4** with **1b** (vide infra). In the case of **5c**, catalysis by *N*-methylimidazole buffers is larger at pH 7.4 than at pH 8.4 by a factor of 3 signifying that the acidic form of the buffer is the catalytically active one.

3. The process given in Scheme III requires for ammonium thiolates in which the pK_a of SH and NH^+ both fall in the range of neutrality, that the k_2^{obsd} vs pH profile should be bell-shaped. It should reach a maximum at the pH where the zwitterionic form is optimized and fall with further increases in pH that titrate the essential $N-H^+$. This is not observable for amino thiols **5a–d** because the pK_a^{NH} is >10, and we cannot follow the reaction at larger pH values because the background hydrolysis is too fast. However, for **1b**, exactly the predicted situation occurs, the observed profile being presented in Figure 1. Also in that figure is the NLLSQ best fit line calculated on the basis of eq 3 which in turn is derived from the process shown in Scheme II under the assumption that only the zwitterionic form is the active one.

(a) Different Catalytic Requirements for Attack of Ammonium Thiolates on *p*-Nitrophenyl Acetate and Amide 4. Also shown in Figure 1 is the observed pH/rate profile for attack of **1b** on pNPA at $T = 37$ °C, $\mu = 0.3$ (KCl).[9] The plot fits the kinetic expression in eq 4 which is derived for the

$$k_2^{obsd} = \frac{(k_2' K_a^{Im} K_{ZW})[H_3O^+] + k_2'' K_a^{Im} K_a^{SH}}{[H_3O^+]^2 + (K_a^{Im} + K_a^{Im} K_{ZW})[H_3O^+] + K_a^{Im} K_a^{SH}} \quad (4)$$

process in Scheme II wherein both the zwitterionic and anionic thiolate are active.[9] Hupe and Jencks[28] have shown that the attack of basic thiolates on substituted phenyl acetates shows a small dependence on thiol basicity ($\beta_{NUC} = 0.27$) and a break near $\Delta pK_a = 0$ to a slope of $\beta_{NUC} = 0.84$ as the pK_a of the thiol is decreased and phenolate expulsion becomes rate limiting. This is the expected situation for a two-step process in which the RDS changes from attack (k_1) to uncatalyzed breakdown of the intermediate (k_2, eq 5). Indeed, this seems to be the case for the attack of

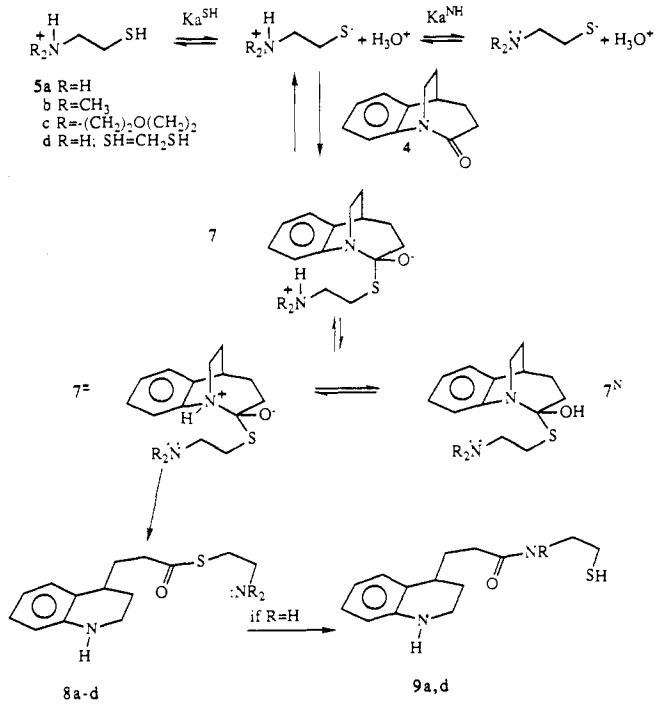


(5)

1b on **4** since the more basic thiolate form (the anionic one) is more active than the less basic zwitterionic one. Here, the RDS is simply S^- attack and *p*-nitrophenoxide expulsion is fast and requires no following general acid catalysis. The presence of the imidazole is passive, serving only to modify the SH pK_a depending upon its existence as $Im-H^+$, or Im.

On the other hand, the bulk of the evidence we have presented for amino thiol attack on amide **4** suggests that thiolate attack is followed by $N-H^+$ trapping of the unstable intermediate **7**, Scheme III). Shown in Figure 2 is a plot of the maximal k_2 values for attack of the various thiols (**1b**, **5a–d**) on **4** vs their pK_a^{SH} values in H_2O and D_2O. Two main points are evident. First, the data cannot be reconciled by any simple Brönsted relationship, which implies that the rate constants are not simply dependent upon the thiolate basicity (nucleophilicity). Admittedly, **1b**, **5a–c**, and **5d** could be members of three sets of amino thiols, each adhering to its own Brönsted line. Second, although there is a small solvent isotope effect of ~0.4 units[23] on the pK_a^{SH} values, the $(k_2)_{H/D}$ ratios are, in all cases, indistinguishable from unity (actually 1.0 ± 0.1). Since the preferred mechanism involves the essential steps of S^- attack and $N-H^+$ trapping, it might be supposed that amino thiols whose structure impedes the following trapping should be less reactive than expected on the basis of the thiol pK_a. For example, the 3-aminopropanethiol derivative (**5d**) has the highest pK_a^{SH} of the series but contains a three carbon link with more rotational degrees of freedom that would have to be restricted in

Scheme III



transferring the the NH^+ proton to the tetrahedral intermediate. Consistent with this, its k_2 value is far lower than the value for cysteamine. In this, and related cases, the requisite proton transfer might be anticipated to become part of the rate-limiting step and therefore subject to a solvent kie: this is not observed. Although the observed values of unity are lower than what is customarily expected for a rate-limiting general catalyst mediated proton transfer,[29] they do not rule out our preferred mechanism. Analysis of solvent isotope effects is complex,[29,30] and small values have been explained by invoking nonlinear N–H–X geometries, asymmetric transition states, and solvation effects, any of which could be operative in the reactions in question. Furthermore, there has been a long-standing debate whether the proton transferred by a general catalyst would show a primary isotope effect of unity (solvation rule[31]) or a more normal effect with a potential maximum value based in the usual way on the vibrational frequency of the bond from H to catalyst.[30b,32] Kresge[33] and Jencks[34] have demonstrated very narrow isotope maxima for general-base-catalyzed reactions, and these were for trapping of an unstable intermediate by the catalyst with the actual proton transfer being rate limiting only for a narrow range around $\Delta pK_a = 0$. Outside this range, when the proton transfer is thermodynamically very uphill or downhill, the primary isotope effect would be small. This would be the expected case for uphill proton transfer from the not very acidic ammonium ions of **5a–d** to the anilino N of **7**, where the pK_a of its corresponding ammonium is roughly 5–6.[25] Even if, as mentioned before, trapping involved a more thermodynamically neutral $N-H^+-O$ transfer to generate $\mathbf{7}^N$, a large skie would not be observed if the proton transfer is fast, and some other

(28) Hupe, D. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 451.

(29) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw Hill: New York, 1969; pp 243–281.

(30) (a) Melander, L.; Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules*; Wiley-Interscience: New York, 1980; pp 202–224 and 42. (b) Schowen, R. L. *Prog. Phys. Org. Chem.* **1972**, *9*, 275–332. (c) More-O'Ferrall, R. A. In *Proton Transfer Reactions*; Calden, E. F., Gold, V., Eds.; Wiley: New York, 1975; p 201.

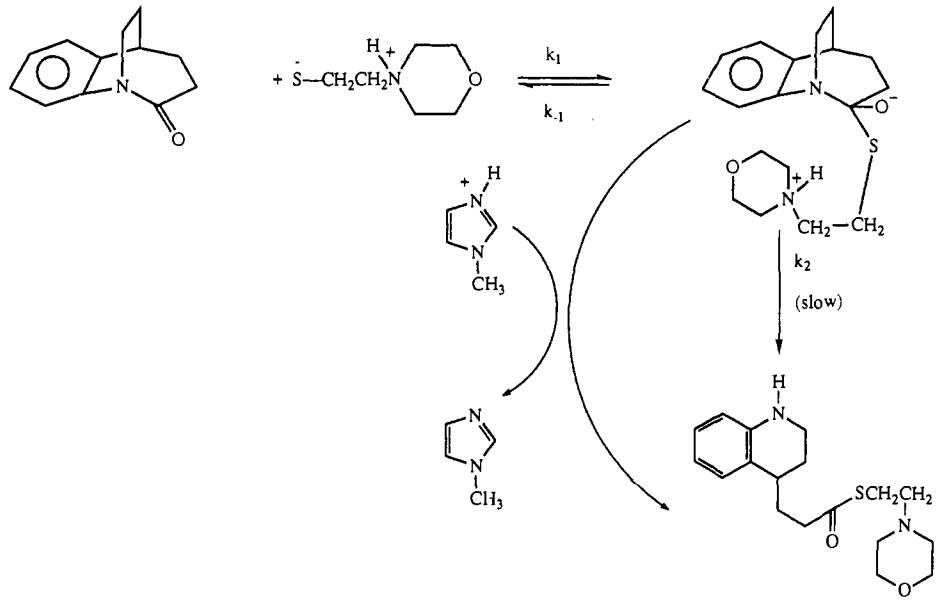
(31) (a) Swain, C. G.; Thornton, E. R. *J. Am. Chem. Soc.* **1962**, *84*, 817. (b) Thornton, E. R. *J. Am. Chem. Soc.* **1967**, *89*, 2915.

(32) (a) Schowen, K. B. J. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978. (b) Schowen, R. L. In *Isotope Effects on Enzyme Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrup, D. B., Eds.; University Park Press: Baltimore, 1977.

(33) Bergman, N. A.; Chiang, Y.; Kresge, A. J. *J. Am. Chem. Soc.* **1978**, *100*, 5954.

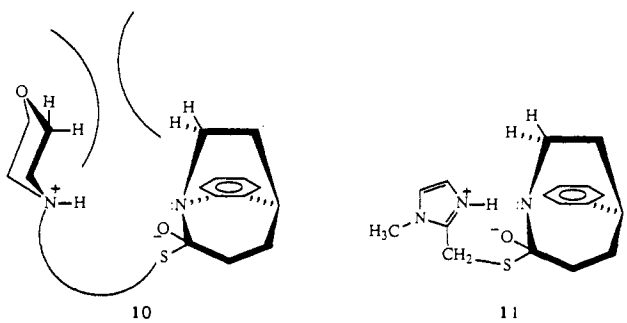
(34) Cox, M. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1981**, *103*, 572.

Scheme IV



step were rate limiting. Based on arguments presented above, $7^{\pm}$ is probably the form required for breakdown to product so that if 7^{N} were formed, the intramolecular catalyst could be envisioned as the base that assists in transferring the proton from the oxygen to the anilino N. In effect, the overall process is equivalent to attack of the zwitterion on **4** followed by proton transfer to yield $7^{\pm}$.

A comparison of the attack of **1b** and **5c** is instructive since both have similar pK_a values for the ionization of the SH to form the zwitterion (e.g., 6.48 (Table II) and 6.44 (Table I), respectively). However, as shown in Figure 2, the maximal k_2 values differ by ~2 orders of magnitude with the imidazole derivative having a k_2^{max} of ~100 M^{-1} s^{-1}. For the two tetrahedral intermediates (**7**, Scheme III) produced from attack of the zwitterions on **4**, proton transfer from the morpholine-H^+ to the aniline is more thermodynamically uphill than is the case for **1b**. Furthermore, inspection of molecular models for **7** reveals that for the morpholino derivative the following proton transfer to create $7^{\pm}$ may be sterically inhibited because the N–H^+ proton must be transferred perpendicular to the six-membered morpholine ring. This introduces severe steric compression between that ring and the bicyclic anilide portion, e.g., **10**. However, for the corresponding tetrahedral intermediate generated from attack of **1b** (e.g., **11**), the N–H^+ proton is transferred in the plane of the imidazole ring, which avoids the steric buttressing apparent in **10**.



10 11

If proton transfer is hindered in the case of the morpholine derivative, the reaction may show evidence of catalysis by added general acids. Shown in Figure 3 is a plot of the observed second-order rate constant for attack of **5c** on **4** at pH 7.4 and 8.4 as a function of added *N*-methylimidazole (NMI). Also in the plot is shown the effect of [NMI] on the rate of attack of **1b** at pH 7.3. Two things are apparent. First, with **1b**, added NMI, up to a concentration of 0.5 M, does not change k_2. This rules out any general acid catalytic effect on initial attack of RS^- and suggests that external buffers cannot compete with an already efficient following intramolecular catalysis. Second, in the case of **5c**, increasing [NMI] up to 2 M accelerates the reaction markedly. We had hoped that eventually, when the following proton transfer was no longer rate limiting, that saturation kinetics would be observed, and further increases in [NMI] would produce no acceleration. Unfortunately, we were limited to [NMI] $\leq$ 2 M which is apparently less than is required to change the rate limiting step. Finally, that the slope of the line at pH 8.4 is less than that at pH 7.4 indicates that the following catalysis is of a general acid nature. Overall, the process consistent with these observations is shown in Scheme IV.

Conclusions and Relevance to the Cysteine Proteases. The general features of the model system of relevance to the acylation of papain by *p*-nitrophenyl esters or by anilides are the pH vs log k_2^{obsd} profiles shown in Figure 1. On the basis of kinetics of acylation of the enzyme by a series of para-substituted phenyl esters of hippuric acid, a Hammett ρ of +1.2 was observed.[3c] Since the phenoxides are relatively good leaving groups, the positive ρ suggested that formation of the tetrahedral intermediate was rate limiting,[3c,i] and expulsion of the leaving group was fast in all cases and required no general acid catalysis. However, when the leaving group becomes poorer, as in the case of the para-substituted anilides of hippuric acid, a Hammett ρ of −1.04 is obtained for the papain mediated hydrolyses.[4c] This change in ρ is consistent with the breakdown of the intermediate being rate-limiting for anilide hydrolyses and suggests that general acid catalysis of these leaving groups is necessary.

The model system of acylation of **1b** by pNPA or **4** shows exactly the same trend. For the ester, the plot in Figure 1 shows that the thiolate nucleophilicity alone dictates the reaction rate and that the state of ionization of the pendant imidazolium is only important insofar as it modifies pK_a^{SH}. For attack on **4**, the available evidence suggests that both thiolate nucleophilicity and subsequent general acid trapping of the unstable tetrahedral intermediate are important in the two step process. The bell-shaped dependence of the rate constant on pH is readily accommodated in the high pH range by titration of the essential imidazolium N–H^+.

The data for acylation of **1b** or **5c** by **4** reveal special features about the imidazole that make it ideally suited for enzymatic catalysis. The first, which is often cited, is that because its conjugate acid form has a pK_a of 7, or close to it depending upon the environment, the imidazole is an ideal mediator of biological proton transfer because it can act as an acid or a base at physiological pH. Moreover, for H^+-transfer from a catalytically

essential imidazolium to the unstable tetrahedral intermediates produced from amides where the N is an aliphatic one, the proton transfer is thermodynamically favorable, and in the case of anilides is either thermodynamically neutral or only slightly uphill.

The second and less obvious feature of imidazole which makes it unusually suitable for general-acid or -base catalysis relative to an aliphatic amine (ammonium) has to do with the molecular shape. Because the proton transfer in imidazole occurs at the sp^2 N, and in the plane of the ring, there is minimal steric hindrance in transfer to sterically congested tetrahedral intermediates.

Acknowledgment. The authors gratefully acknowledge the financial support of the University of Alberta and the Natural Sciences and Engineering Research Council of Canada. In addition, J.K. acknowledges NSERC for a Post Graduate Scholarship.

Supplementary Material Available: Tables of observed rate constants vs pH for attack of thiols **1b**, **5a-d**, and **6a-d** on amide **4** and characterization of thiol ester **8b** and amide **9a** (7 pages). Ordering information is given on any current masthead page.

Chemistry and Structure of Modified Uridine Dinucleosides Are Determined by Thiolation

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Abstract: The structural determination of modified nucleosides is important for understanding the chemistry, structure, and functional changes that they introduce to the nucleic acids in which they occur. Thiolation of transfer RNA wobble position uridine produces an energetically stabilized conformation of the nucleoside in solution at ambient temperature that is independent of the nature of the 5-position substituent and is of biological significance to tRNA selection of only those codons ending in adenosine (Sierzputowska-Gracz, H.; Sochacka, E.; Malkiewicz, A.; Kuo, K.; Gehrke, C.; Agris, P. F. *J. Am. Chem. Soc.* **1987**, *109*, 7171-7177. Agris, P. F.; Sierzputowska-Gracz, H.; Smith, W.; Malkiewicz, A.; Sochacka, E.; Nawrot, B. *J. Am. Chem. Soc.*, in press). Dinucleoside monophosphates have been synthesized as models for investigating the conformations and structures of wobble position uridine-34 that is thiolated and differently modified at position-5 and that is either 3′-adjacent to the invariant uridine-33 in tRNA or 5′-adjacent to the second anticodon position uridine-35. The structures and conformations of 11 dinucleoside monophosphates were analyzed by ^1H, ^{13}C, and ^{31}P magnetic resonance (NMR) spectroscopy. Within the dinucleosides, the individual modified uridine structures and conformations were very similar to those of their respective mononucleosides. The 2-position thiolation, and not the 5-position modification, produced a significantly more stable, C(3′) endo, gauche$^+$, anti conformer. However, within those dinucleosides in which the 2-thiouridine was 5′ to the unmodified uridine, the nucleic acid backbone torsion angles of the unmodified uridine 5′-phosphate were affected, as determined from the scalar coupling constants J_{H^1H}, $J_{H^{31}P}$, and $J_{^{13}C^{31}P}$. In contrast, uridines that were only 5-position modified did not affect the conformation of the 3′-adjacent unmodified uridine phosphate. The structural data obtained and the nucleoside conformations derived from the data support the "modified wobble hypothesis" (Agris, P. F. *Biochimie* **1992**, *73*, 1345-1349); i.e., the tRNA wobble position-34 nucleoside is modified in such a way as to constrain not only its own conformation but also the structural conformation of the anticodon, thereby producing a specific codon selection during protein synthesis.

Introduction

Uridines (U) in the first position, or wobble position-34, of eucaryotic and procaryotic transfer RNA (tRNA) anticodons that are specific for such amino acids as glutamine, glutamic acid, and lysine are many times found to be naturally thiolated at C(2).[1-4] Thiouridine-34 (s^2U) is almost always found to be additionally modified at C-5.[2] There are a variety of naturally occurring 5-position derivatives: (methylamino)methyl, methoxy, (methoxycarbonyl)methoxy, (methoxycarbonyl)methyl, [(carboxymethyl)amino]methyl, and others.[5] The structures and conformations of the 2-thiolated, 5-derivatized uridines at ambient temperature are dictated by the presence of the thio group and barely influenced by the chemical or structural nature of the 5-substituent.[6] Therefore, all 2-thiouridines have been found to be predominantly anti, C(3′) endo, gauche plus,[6,7] whereas nonthiolated 5-position derivatives can be either syn or anti and have no significant preference for either the C(3′) endo or C(2′) endo conformations.[7] The 2-thio-5-derivatized uridines are thermodynamically more stable than their nonthiolated counterparts.[8]

As an explanation of the codon selectivity exhibited by tRNAs with s^2U, its derivatives, and certain other wobble position modified nucleosides, we have offered a "modified wobble hypothesis" based

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0002-7863/92/1514-7989$03.00/0