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Formation of N-Substituted 2-Iminothiolanes When Amino Groups in Proteins and Peptides Are Modified by 2-Iminothiolane

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The reagent 2-iminothiolane (2-IT) is used to introduce thiol groups into proteins and peptides by reactions of their amino groups. In this study, we report that the thiol adduct initially formed by the reaction of an amine with 2-IT (a 4-mercaptobutyramidine) is unstable and decays by a first-order process to a nonthiol product (an N-substituted 2-iminothiolane) with the loss of ammonia. The thiol adducts derived from amines of low pK_a values (~8; e.g., α-amino groups in peptides) decay more rapidly than those derived from amines of high pK_a values (~9.5; e.g., benzylamine, ethanolamine, lysine residues in proteins), with half-lives at pH 8 ranging from 0.3 to 3 h at 23°C, and from 1 to 44 h at 0°C. In the case of reactions of peptides with 2-IT, the substituents at the α-carbon also influence the decay of the initial thiol adducts. The decay of the initial thiol adduct to an N-substituted 2-iminothiolane was confirmed for the reaction between benzylamine and 2-IT by the isolation of *N*-benzyl-2-iminothiolane and its characterization by elemental analysis and mass spectrometry. The decay of the initial 4-mercaptobutyramidine is prevented if the thiol group is capped, e.g., in the form of a disulfide group, or if the solution is acidified (pH 3 to 4). Immediate capping of the thiol is, therefore, recommended when using 2-IT in the formation of bioconjugates. For amines of high pK_a, the N-substituted 2-iminothiolane product can be cleaved by hydroxylamine, resulting initially in a thiol which then decays to *N*-hydroxy-2-iminothiolane regenerating the original amine. For amines of low pK_a, the N-substituted 2-iminothiolane product can be hydrolyzed at pH 5 to generate a stable thiol with an amide functionality (an N-substituted 4-mercaptobutyramide). © 1996 Academic Press, Inc.

The reagent 2-iminothiolane (2-IT)[3] is used to incorporate thiol groups into proteins by reactions of their lysine amino groups (Scheme 1) (1, 2). Modification of amino groups with 2-IT, which was first described as methyl 4-mercapto-butyrimidate (3), results in positively charged amidine derivatives. Thus, when 2-IT is applied to the modification of lysine residues in proteins, the surface charge distribution of the protein is unperturbed (1). The thiol groups incorporated by 2-IT have been used to cross-link proteins by a reversible disulfide linkage in multiprotein complexes such as ribosomes (4–8). Such thiol derivatives from a variety of substrates bearing primary amino groups (e.g., proteins, peptides, and amino sugars) have also been used in conjugation reactions with other molecules bearing electrophilic groups such as iodoacetamido or 4-dithiopyridyl groups (9–11). 2-IT has been used to introduce thiol groups into the cytotoxic protein gelonin for conjugation via a disulfide linkage to antibodies or interleukin-2 (12, 13). Several substituted 2-iminothiolanes (5- and 4,5-alkyl substituted) have been used to modify antibodies in the presence of the reactive disulfide 5,5′-dithio-bis(2-nitrobenzoic acid) resulting in activated disulfide groups that were reacted with the thiol group in ricin A chain to prepare conjugates with hindered disulfide linkages (14).

A 2-IT-modified glycopeptide ligand comprising a triantennary N-linked oligosaccharide was used to chemically block the galactose-binding sites in ricin (15). An immunotoxin has been prepared by conjugation of this

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[3] Abbreviations used: 2-IT, 2-iminothiolane; bis-tris, bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane; BSA, bovine serum albumin; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PDE, 2-(2-pyridyldithio)ethanol; SPDP, *N*-succinimidyl 3-(2-pyridyldithio-)propionate; TEA, triethanolamine; TLC, thin layer chromatography; UV, ultraviolet; ΔA, change in absorbance; δ, NMR chemical shift in ppm (parts per million).

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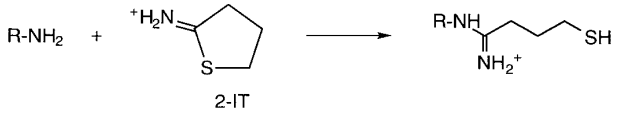


SCHEME 1

modified ricin with an antibody (16, 17). It was presumed that the 2-IT-modified glycopeptide moiety in this immunotoxin had an amidine functionality. In another study, however, where 2-IT was reacted with the α-amino group on an asparagine residue of a glycopeptide, analysis by mass spectrometry identified the thiol product as having an amide group and not the expected amidine function (18). In a different study using mass spectrometry, the reaction products of 2-IT and α-amino groups in peptides were shown to contain the expected amidine derivatives together with a second type of products of masses 17 units lower than the amidine derivatives. These by-products were proposed to have arisen from a cyclization reaction with the loss of ammonia because they were not observed when the initial thiol products were derivatized *in situ* using *N*-pyrenylmaleimide (19). These reactions of α-amino groups in peptides with 2-IT, which were carried out at room temperature or at 37°C, apparently resulted in side reactions that have not been reported for the modifications of lysine groups in proteins with 2-IT, which are typically carried out at 0°C.

We describe in this report the hitherto unrecognized fact that the thiol adducts initially formed by the reaction of amines with 2-IT are inherently unstable and decay with loss of ammonia to non-thiol products. We have identified the non-thiol products as N-substituted 2-iminothiolanes and propose a mechanism for the initial formation of thiol followed by its decay. We have studied the influence of the pK_a of the reacting amino group, of the nature of α-substituents of peptides, and of the reaction temperature on the type of final reaction product obtained in the reaction of 2-IT and amine. Awareness of the potential for secondary reactions by the 4-mercaptobutyramidine functionality is necessary for the proper design of experiments which utilize this valuable reagent. Recommendations regarding the biochemical applications of 2-IT are discussed.

EXPERIMENTAL SECTION

Materials

2-Iminothiolane hydrochloride (2-IT · HCl), ethanolamine hydrochloride, glycinamide hydrochloride, glycylglycine, L-aspartyl-L-phenylalanine amide, L-asparaginamide, L-alanine, L-alanyl-L-alanine, L-tyrosinyl-L-glycyl-L-glycine, 2-(2-pyridyldithio)ethanol hydrochloride (PDE), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). L-Aspartyl-L-phenylalanine, L-aspartyl-L-phenylalanine methyl ester, 4-aminomethylbenzoic acid hydrochloride, γ-thiobutyrolactone, dithiothreitol, deuterium oxide, deuterated methanol (CD_3OD), sodium deuteroxide (40% by weight in D_2O), and deuterium chloride (37% by weight in D_2O) were from Aldrich (Milwaukee, WI). Benzylamine was distilled from commercial benzylamine (Aldrich). L-Asparaginyl-L-valine was from Bachem Bioscience Inc. (Philadelphia, PA). All buffers were prepared using analytical grade reagents.

Determination of Half-Lives of Thiol Adducts of Amines and 2-IT

A reaction mixture initially containing 22 mM amine, 5 mM 2-IT, and 5 mM EDTA in 200 mM triethanolamine buffer (TEA buffer), pH 8, was kept at 23°C (or at 0°C), and aliquots of the reaction mixture were assayed for thiol content by Ellman's assay at several time intervals. The assay mixture contained a 20-μl aliquot of the reaction mixture, 970 μl of 50 mM sodium phosphate buffer, pH 7, containing EDTA (1 mM), and 10 μl of a 100 mM DTNB solution in DMSO. The change in absorbance at 412 nm (ΔA_{412}) was measured. The rate constant (k) for decay of the initial thiol adduct was calculated from the plot of ln ΔA_{412} vs time, which included the time points after the maximum value of ΔA_{412} was reached (Fig. 1B). The half-life of the initial thiol adduct was calculated from the rate constant of its decay using the first-order decay equation: $t_{1/2} = 0.693/k$. The extinction coefficient at 412 nm for Ellman's assay was assumed as 14150 M^{-1} cm^{-1} (20).

HPLC Analysis of Reaction of Ethanolamine with 2-IT

The reaction of ethanolamine with 2-IT was analyzed using reversed-phase HPLC (C-18 Alltima Column, 250 × 4.6 mm; Alltech). A reaction mixture initially containing 90 mM ethanolamine and 5 mM 2-IT at pH ~8, and a 2-IT control solution initially containing 5 mM 2-IT at pH ~8 were kept at 28°C. At various time intervals, 0.4-ml aliquots of the reaction mixture or the 2-IT control were diluted using 1.2 ml of 50 mM potassium phosphate buffer, pH 3.2. The diluted solutions were then used for injection (20 μl) into HPLC (1 ml/min flow rate, 245 nm detection) using a premixed 99:1 (v/v) mixture of 50 mM potassium phosphate buffer, pH 3.2, and acetonitrile as eluant (Fig. 2A).

Isolation of Non-thiol Product from the Reaction of Benzylamine with 2-IT

A solution of benzylamine (0.54 ml, 5 mmol) and 2-IT · HCl (0.34 g, 2.5 mmol) was prepared in 50 ml water,

and its pH was lowered to ~7 by adding 3 ml of 1 N HCl. The solution, which was initially clear, started becoming turbid after about 3 h of stirring at ambient temperature. After 21 h of stirring, oily droplets were seen in the reaction mixture. The oily droplets extracted into ethyl acetate (2 × 60 ml) as insoluble floating droplets. The ethyl acetate extract (120 ml) became clear on addition of 10 ml methanol. Silica gel TLC, using a 20:1 (v/v) mixture of ethyl acetate and methanol as eluant, showed a major spot at R_f 0.63 (detected in UV light or by iodine staining). The ethyl acetate:methanol solution was concentrated on a rotary evaporator and then purified by silica gel chromatography using a 20:1 (v/v) mixture of ethyl acetate and methanol as eluant. The fractions containing the desired product were concentrated on a rotary evaporator and then dried on a vacuum pump, yielding a pale liquid (0.20 g). ^{1}H NMR spectrum (60 MHz, CD_3OD): δ 7.4 (s, 5 H, C_6H_5—), 4.45 (s, 2 H, C_6H_5—CH_2—), 3.3 (t, J = 6.4 Hz, 2 H, —C(=NR)—CH_2—CH_2—CH_2—S—), 2.7 (t, J = 7 Hz, 2 H, —C(=NR)—CH_2—CH_2—CH_2—S—), 2.2 (m, 2 H, —C(=NR)—CH_2—CH_2—CH_2—S—). The product was also analyzed by elemental analysis (*Anal.* Calcd. for $C_{11}H_{13}NS$: C, 69.07; H, 6.85; N, 7.32. Found: C, 69.06; H, 6.77; N, 7.27), and by mass spectrometry using chemical ionization ([M + H^+] = 192) (Oneida Research Services, Whitesboro, NY).

^{1}H NMR Study of the Reaction of 4-Aminomethylbenzoic Acid with 2-IT

A solution of 4-aminomethylbenzoic acid · HCl (14.2 mg, 94 mmol) and 2-IT · HCl (9.6 mg, 70 μmol) in 0.88 ml D_2O in an NMR tube was treated with 40 μl of 4% NaOD solution in D_2O. The p*D* of the final reaction mixture was ~7.5 (pH paper). ^{1}H NMR spectra of the reaction mixture, which remained clear, were recorded at several time intervals. After 3 days, *tert*-butanol was added as reference (δ 1.22 ppm) and the ^{1}H NMR spectrum was recorded. All NMR spectra were then aligned using internal *tert*-butanol peak as reference (Fig. 3).

Decay of Thiol Groups in a Sample of Bovine Serum Albumin Modified with 2-IT

The decay of thiol groups in a sample of bovine serum albumin (BSA) modified with 2-IT was measured at pH 7 at both 25 and 0°C, and was compared to the decay of thiols in a control sample of BSA which had been modified using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and then reduced with DTT.

A sample of BSA (4.3 mg/ml; previously treated with iodoacetamide to alkylate the native thiol group in BSA) was modified using a 42-fold molar excess of 2-IT in 100 mM sodium phosphate buffer, pH 8, on ice for 1.5 h. The reaction mixture (6 ml) was then acidified to pH ~4–5 using acetic acid and dialyzed at 4°C in 2 liters of 50 mM sodium acetate buffer, pH 4.5, containing 1 mM EDTA, and then in 2 liters of 5 mM sodium acetate buffer, pH 4.5, containing 1 mM EDTA (acetate/EDTA buffer, pH 4.5). This modification resulted in two thiol groups per BSA molecule (M_r (BSA) = 66 000; $A_{1\text{ cm}}^{0.1\%}$ = 0.62 at 280 nm). The dialyzed reaction mixture in acetate/EDTA buffer, pH 4.5, and 200 mM sodium phosphate buffer, pH 7, containing 2 mM EDTA (phosphate/EDTA buffer) were degassed on ice in a vacuum desiccator for 20 min at ~30 Torr, and then on a vacuum pump for 25 min at <1 Torr. The desiccator was filled with argon before it was opened to air. An aliquot of the degassed dialyzed reaction mixture was diluted with an equal volume of degassed phosphate/EDTA buffer, pH 7, and kept at 25°C for 3 h, and its aliquots were analyzed for thiol content by Ellman's assay. Another sample prepared by dilution with phosphate/EDTA buffer, pH 7, was kept on ice for 1 day, and its aliquots were analyzed for thiol content by Ellman's assay.

A control sample of BSA (4.3 mg/ml; iodoacetamide-treated) was modified with a 19-fold molar excess of SPDP in 30 mM sodium phosphate buffer, pH 7, containing 1 mM EDTA, at 30°C for 40 min, and the reaction mixture (4 ml) was dialyzed in 2 liters PBS (10 mM potassium phosphate, pH 7.2, containing 150 mM NaCl) at 4°C. The dialyzed reaction mixture in PBS was treated with 3 mM DTT on ice for 45 min, and was then dialyzed in acetate/EDTA buffer, pH 4.5, as described above for the sample modified with 2-IT. The modification of BSA using SPDP and DTT resulted in 6.9 thiol groups per BSA molecule. The degassed, dialyzed reaction mixture was diluted using an equal volume of degassed phosphate/EDTA buffer, pH 7, and was then analyzed for decay of thiol at 25 and at 0°C as described above for the sample modified with 2-IT. In a separate control experiment it was shown that the native disulfide bonds in unmodified BSA (iodoacetamide-treated; but not treated with SPDP) were not reduced to a significant extent on treatment with 3 mM DTT in PBS at 4°C; only 0.13 thiol groups per BSA molecule were detected.

Determination of pK_a of Amines

The pK_a values of amino groups were determined by pH titration of 50 mM solutions of amines (in protonated form) in water by addition of NaOH.

Determination of pK_a of 2-IT and N-Substituted 2-Iminothiolanes

The pK_a values of 2-IT and several N-substituted 2-iminothiolanes were determined by measuring their absorbances at 248 nm (due to the protonated 2-iminothiolane chromophore) in buffers in the range of pH 3–10. The following 100 mM buffer solutions were used: sodium phosphate (pH 3, 3.5, 6, 6.5, 7, 7.5, 8), sodium acetate (pH 4, 4.5, 5, 5.5), and sodium borate (pH 8.5,

9, 9.5, 10). The background absorbance value at pH 10 was subtracted from all absorbance values, and the ΔA values were plotted as a function of pH (Fig. 4).

Generation of Thiols by Acidic Hydrolysis of the Non-Thiol Products Derived from the Reactions of Amines with 2-IT

The reaction mixtures of several amines (22 mM) with 2-IT (5 mM) in TEA buffer, pH 8, were incubated at ambient temperature for about 1 day. The resulting solutions, which had little thiol content, were then diluted 10-fold using 200 mM sodium acetate buffer, pH 5, containing EDTA (0.5 mM), and the generation of thiol groups was measured by Ellman's assay over a period of 5 days after acidification (Fig. 5).

Preparation of the Thiol Adduct of Glycylglycine and γ-Thiobutyrolactone

Authentic samples of N-substituted 4-mercaptobutyramide (R—NH—CO—CH_2—CH_2—CH_2—SH) were prepared by reactions of amines (R—NH_2) and γ-thiobutyrolactone for comparison with the thiols formed by acidification of the non-thiol products derived from reactions of amines and 2-IT. A solution of glycylglycine (0.145 g, 1.1 mmol) in 5 ml water was added to a solution of γ-thiobutyrolactone (22 μl, 0.25 mmol) in 5 ml ethanol, followed by the addition of 0.1 ml of 0.1 M EDTA solution (pH 7). The reaction was initiated by addition of 1.1 ml of 1 N NaOH, resulting in a pH of ~11 (pH paper). After 50 min of stirring at ambient temperature, another 0.2 ml of 1 N NaOH was added to raise the pH of the solution to 11 again. After 90 min of reaction, the thiol concentration in the mixture was 20 mM by Ellman's assay. The reaction mixture was then acidified to pH 4–5 by addition of HCl.

Another sample prepared in a similar manner as above (but without EDTA) was concentrated on a rotary evaporator to remove ethanol, acidified to pH 1, and extracted with ethyl acetate. The ethyl acetate extract was concentrated on a rotary evaporator and then dried on a vacuum pump, dissolved in D_2O, and its ^1H NMR spectrum was recorded.

Determination of Rate Constants of Reactions of Thiols with N-Ethylmaleimide

To differentiate thiol groups based on their reactivities with *N*-ethylmaleimide (NEM), rate constants were compared for the reaction of NEM with N-substituted 4-mercaptobutyramide (R—NH—CO—CH_2—CH_2—CH_2—SH) and N-substituted 4-mercaptobutyramidine (R—NH—C(=NH_2^+)—CH_2—CH_2—CH_2—SH). An N-substituted 4-mercaptobutyramide was prepared by the reaction of 4-aminomethylbenzoic acid and γ-thiobutyrolactone (similar to the reaction of glycylglycine and γ-thiobutyrolactone described above). An N-substituted 4-mercaptobutyramidine was prepared by the reaction of 4-aminomethylbenzoic acid and 2-IT at 0°C. These thiols were prepared using an excess of amine over γ-thiobutyrolactone or 2-IT, and the reaction mixtures were used in the kinetic experiments without separation of the thiols. The rates were studied at 23°C in 200 mM sodium acetate buffer, pH 5, containing EDTA (0.5 mM), by monitoring the decrease in absorbance of NEM at 302 nm. The concentrations of NEM and thiol in the kinetic experiments were equal; typical initial concentrations were: c = [Thiol] = [NEM] = 0.5 mM. An extinction coefficient $\epsilon_{302\,nm}$ of 620 M^{-1} cm^{-1} was used for NEM (21). Rate constants (k_{app}) were determined using the integrated second-order rate equation: $k_{app} \times t = [(1/c_{final})-(1/c_{initial})]$. These rate constants are apparent rate constants which are based on the apparent concentration of thiol (i.e., the total concentration of thiol and thiolate).

RESULTS

Generation of Thiols by the Reactions of Primary Amines with 2-IT

In the course of modification of a glycopeptide with 2-IT we noticed that the sulfhydryl content declined rapidly, even in solutions containing EDTA. We therefore studied the reactions of several model amines with 2-IT, at both 23 and 0°C, for the generation and the stability of thiol groups using Ellman's assay at different time intervals. The reactions of all amines with 2-IT were fast at pH 8. For example, the reaction of 5 mM 2-IT with 22 mM Ala-Ala at 23°C resulted in the formation of a maximum amount of thiol (i.e., maximum ΔA in Ellman's assay) within 20 min (Fig. 1A). However, the thiol content of the reaction mixture then decreased with time. This decay of thiol was found to be the same in the presence or absence of EDTA, which indicated that it was not due to oxidation. The plot of ln ΔA vs time was linear with a negative slope indicating a first-order decay process. The decay curves for the thiol adduct of Ala-Ala are shown in Fig. 1B and were used to calculate the half-life of 44 min at 23°C and 10 h at 0°C. The half-lives of the initial thiol adducts of other amines and 2-IT were similarly determined and are listed in Table 1.

For the reactions of 2-IT with a variety of amines ranging in pK_a values from 7.6 to 9.7, the calculated half-lives of the initial thiol adducts range from 0.3 to 3 h at 23°C, and from 1 to 44 h at 0°C (Table 1). The thiol adducts derived from α-amino groups in peptides (pK_a ~8) decayed faster than those derived from amines of higher pK_a (~9.5) such as benzylamine and ethanolamine. Within the set of peptides analyzed, the reaction of the α-amino groups with 2-IT and the decay of the thiol adducts were found to be influenced by the substituents at the α-carbon (results shown later).

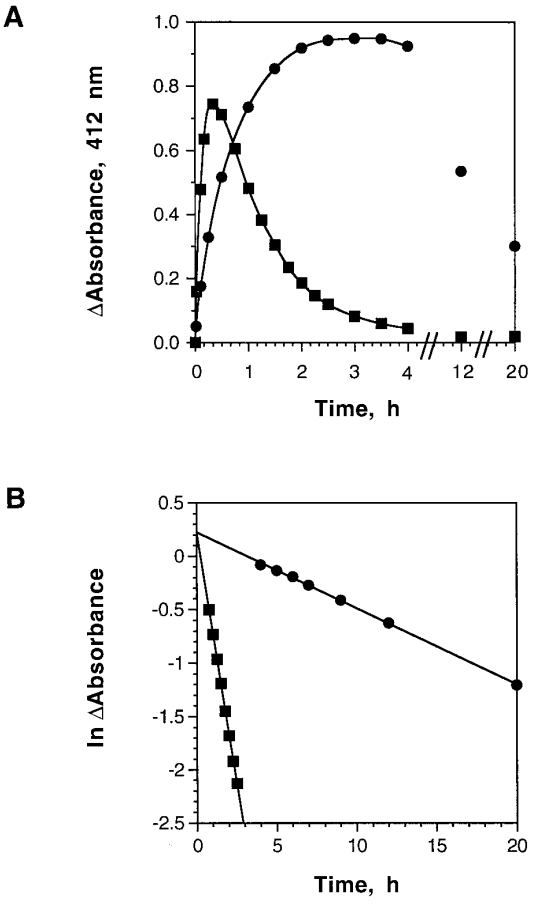


FIG. 1. Assay of thiol in reaction mixtures of Ala-Ala and 2-IT at 23°C (■) and at 0°C (●). The intial concentrations were: [Ala-Ala] = 22 mM, [2-IT] = 5 mM; [EDTA] = 5 mM; pH 8. Samples were taken at intervals and diluted 50-fold into Ellman's assay solution. (A) Plot of ΔA (Ellman's assay) vs time. (B) Plot of ln ΔA vs time, plotted for the time points after the maximum thiol had been generated. The theoretical value of ΔA was 1.41 absorbance unit based on the weight of 2-IT, assuming its complete conversion to the thiol adduct.

We compared the stability of the initial thiol adducts in reactions of amines and 2-IT at both 23 and 0°C (Table 1), since modifications of amino groups of proteins with 2-IT are typically carried out at 0°C, while the modifications of peptides for which unexpected results were reported were performed at ambient temperature (18, 19). As expected, the initial formation of thiol compounds was slower for the reaction at 0°C than for the reaction performed at 23°C. For example, the maximum amount of thiol is formed in about 2.5 h for the reaction of Ala-Ala with 2-IT at 0°C, while it takes only 0.3 h to reach the maximum at 23°C (Fig. 1A). The half-lives of the initial thiol adducts of amines with 2-IT at 0°C are 10- to 20-fold longer than at 23°C with the exception of the fetuin-derived glycopeptide among the amines studied, which has a half-life only 2-fold longer at 0°C than at 23°C (Table 1).

HPLC Analysis of the Reaction of Ethanolamine with 2-IT

Reversed-phase HPLC, with detection at 245 nm for the protonated 2-IT chromophore, was performed under acidic conditions (pH 3.2) to follow the reaction of 2-IT with ethanolamine. In preliminary experiments, the stability of the initial thiol adduct at low pH was studied by acidifying the reaction mixture of ethanolamine and 2-IT to pH 3-4 after 15 min of reaction at pH 8. The acidified reaction mixture showed no significant change in thiol content by Ellman's assay (<3%) over 100 min at ambient temperature. The initial thiol adduct is therefore stable in solutions at acidic pH values of ~3–4.

The HPLC chromatograms of a reaction mixture initially containing 90 mM ethanolamine and 5 mM 2-IT (pH ~8, 28°C) and a control containing 5 mM 2-IT were compared at several time intervals (Fig. 2A). The chromatogram of the 2-IT control showed a 2-IT peak with a retention time of 4 min. This 2-IT peak was unchanged for over 100 min of incubation of the 2-IT control solution. The HPLC chromatogram of the reaction

TABLE 1

Comparisons of Half-Lives of Initial Thiol Adducts of Amines and 2-Iminothiolane at 23 and 0°C[a]

Amine	pK_a	Half-life (h) 23°C	Half-life (h) 0°C
Ethanolamine	9.5[b]	2.8	44
Alanine	9.7[b]	2.5	34
Benzylamine	9.3[b]	1.8	33
Asparaginamide		1.4[d]	—[e]
Asn-Val		1.1[d]	—[e]
Gly-Gly	8.2[c] (8.0)[b]	1.1	22
Glycinamide	8.0[c] (7.9)[b]	1.0	18
Tyr-Gly-Gly		0.8	9
Ala-Ala	8.2[c]	0.7	10
Glycopeptide (fetuin-derived)[f]		0.5	1.0
Asp-Phe	7.9[c]	0.3	4.0
Asp-Phe methyl ester		0.3	3.3
Asp-Phe amide	7.6[c]	0.3	3.6

[a] The reaction mixture of amine and 2-iminothiolane (2-IT) was incubated at 23 or 0°C at pH 8. The initial concentrations in the reaction mixture were typically: [amine] = 22 mM, [2-IT] = 5 mM, [EDTA] = 5 mM. The concentration of thiol in the reaction mixture was determined using Ellman's assay at several time intervals. The half-life of the thiol adduct was calculated from the slope of the plot of ln [absorbance (Ellman's assay)] vs time.

[b] pK_a values from Means, G. E., Congdon, W. I., and Bender, M. L. (1972) *Biochemistry* 11, 3564–3571.

[c] pK_a values determined in this study as described under Experimental.

[d] The maximum value of absorbance in Ellman's assay was about 30% of that expected.

[e] Values were not determined.

[f] The glycopeptide containing triantennary N-linked oligosaccharide (monodesialylated form) was obtained from fetuin (15).

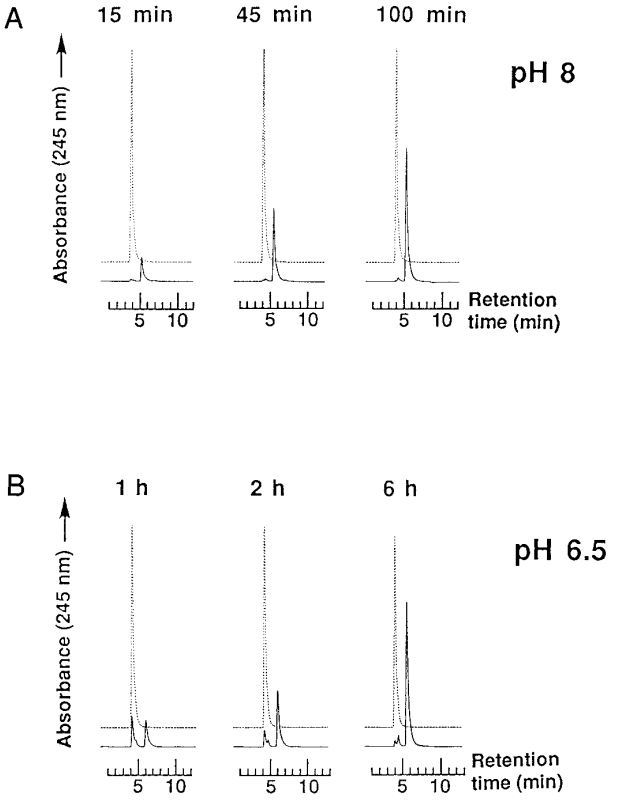


FIG. 2. HPLC analysis of a reaction mixture of ethanolamine and 2-IT. (A) The initial concentrations in the reaction mixture (pH 8) were: [ethanolamine] = 90 mM, [2-IT] = 5 mM. The control sample contained 5 mM 2-IT. At several time intervals, aliquots of the reaction mixture (solid line) and the 2-IT control sample (dotted line) were analyzed using reversed-phase HPLC (pH 3.2; detection at 245 nm; see Experimental). The 2-IT peak eluted at 4 min, and the major peak in the reaction mixture eluted at about 5.4 min. (B) The initial concentrations in the reaction mixture (pH 6.5) were: [ethanolamine] = 100 mM, [2-IT] = 50 mM. A second reaction mixture (pH 6.5) was similar to the above reaction mixture but also contained 200 mM DTT. The HPLC chromatograms for the reaction mixture without DTT (solid line) were identical to those of the reaction mixture with 200 mM DTT (not shown). The control sample (dotted line) contained 50 mM 2-IT.

mixture after 15 min of reaction did not show any peak at the 2-IT position, demonstrating the complete reaction of 2-IT within 15 min. Instead, a single new peak with a retention time of about 5.4 min was seen in the reaction mixture. This peak was small at 15 min and grew upon longer incubation (Fig. 2A). A secondary product having absorbance at 245 nm is therefore slowly formed in the reaction mixture on prolonged incubation.

In another experiment, a mixture of ethanolamine and 2-IT, after 7 min of reaction resulting in a maximum amount of thiol, was then treated with a twofold molar excess of 2-(2-pyridyldithio)ethanol (PDE) to protect the initial thiol adduct as a disulfide. HPLC of the resulting solution showed a small peak of the secondary product at 5.3 min, which did not increase on further incubation of the PDE containing solution. The disulfide derivative of the primary product (resulting from the reaction of the initial thiol adduct with PDE) is therefore stable at ambient temperature.

A possible explanation for the decay of the initial thiol adduct formed in the reaction of amine with 2-IT may be its oxidation. To minimize the loss of thiol due to oxidation, a reaction mixture of ethanolamine (100 mM) and 2-IT (50 mM) was incubated at a lower pH 6.5 in the presence of 200 mM dithiothreitol (DTT), and was compared to a control reaction without DTT (Fig. 2B). This reaction of ethanolamine and 2-IT at pH 6.5 was similar to that at pH 8 (described above) but slower, as expected (Fig. 2B). The presence of 200 mM DTT had no effect on the rate of disappearance of 2-IT or on the rate of formation of the secondary product. In these HPLC runs, fractions were collected at 1-min intervals from 4.5 to 11.5 min, and were analyzed by Ellman's assay. DTT eluted earlier and was not present in these fractions. The 7.5- to 8.5-min fraction contained the maximum amount of thiol. The secondary product, which elutes at ~6 min, is therefore not a thiol. The total thiol assayed in the HPLC fractions was similar for the reaction mixture with DTT and that without DTT, when compared after similar times of incubation, and decreased by ~50% from the 2-h run to the 6-h run. The decay of the initial thiol adduct of ethanolamine and 2-IT must occur by a nonoxidative mechanism because it is unaffected by the presence of both DTT and EDTA.

^{1}H NMR Study of the Reaction of 4-Aminomethylbenzoic Acid with 2-IT

To analyze the product formed by decay of the initial thiol adduct, we followed the reaction of 2-IT with 4-aminomethylbenzoic acid by ^{1}H NMR analysis. A reaction mixture of 2-IT and an excess of 4-aminomethylbenzoic acid (Ar—CH_2—NH_2) in D_2O at pD ~8 was incubated at ambient temperature, and its ^{1}H NMR spectra were recorded at several time points (Fig. 3B). After 20 min, when the initial reaction was complete as measured by the amount of thiol formed, the NMR spectrum showed major peaks at chemical shifts compatible with the amidine thiol (Ar—CH_2—NH—C(=NH_2^+)—CH_2—CH_2—CH_2—SH): δ 4.5 (s, Ar—CH_2—), 2.6 (m, —C(=NH_2^+)—CH_2—CH_2—CH_2—SH) and 2.1 (m, —C(=NH_2^+)—CH_2—CH_2—CH_2—SH) (Fig. 3B). Within 3 h, these peaks largely disappeared and were replaced by novel peaks of the non-thiol product: δ 4.4 (s, Ar—CH_2—), 3.3 (t, —C(=NR)—CH_2—CH_2—CH_2—S—), 2.6 (m, —C(=NR)—CH_2—CH_2—CH_2—S—), and 2.15 (m, —C(=NR)—CH_2—CH_2—CH_2—S—). The peak at δ 2.6 disappeared on prolonged incubation, presumably because of exchange of the —C(=NR)—CH_2— protons with deuterium through imine–enamine tautomerism in D_2O causing

the peak at δ 2.15 to become a sharp triplet (Fig. 3B). The NMR spectrum obtained after 3 days was identical to that obtained after 1 day, indicating that the non-thiol product was stable at pH 8 in the presence of amine. This NMR spectrum of the non-thiol product was similar (but not identical) to that of 2-IT. Note that the triplet at δ 3.5 due to 2-iminothiolane (Fig. 3A) is virtually absent after 15 min of reaction, but a new triplet at δ 3.3 is again prominent after 1.3 h of reaction (Fig. 3B). The spectrum of 2-IT at pH 8 shown in Fig. 3A was recorded 15 min after preparation of the sample;

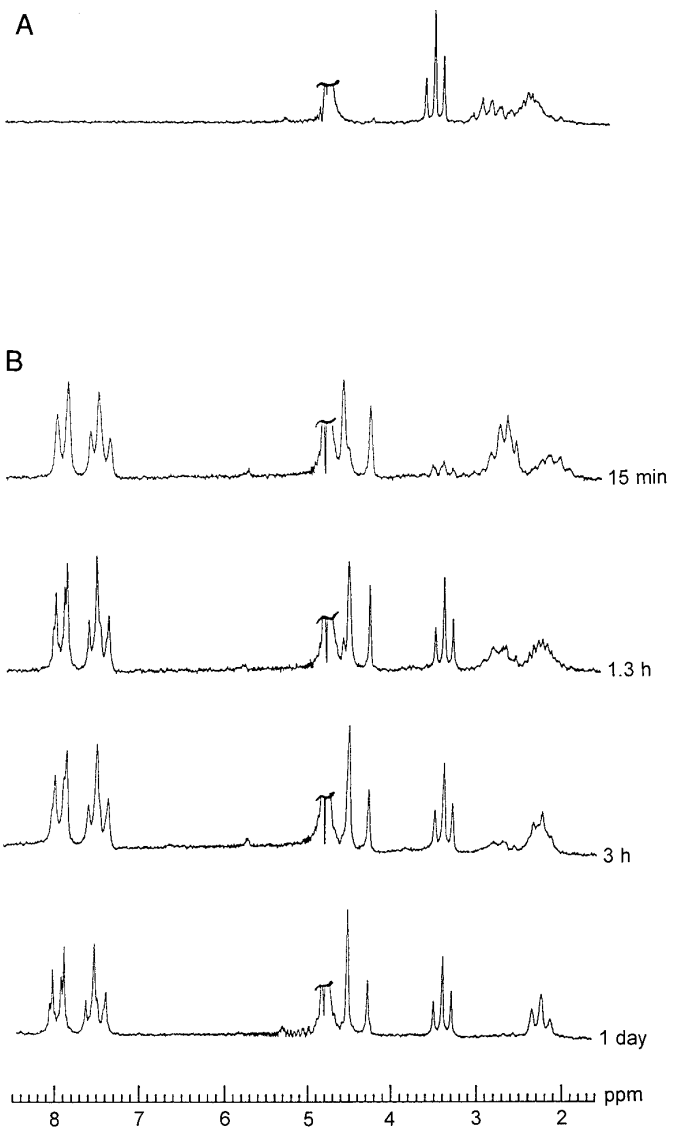


FIG. 3. 60 MHz ^{1}H NMR spectra of (A) 2-IT (0.2 M) in D_2O (p*D* ~8); (B) reaction mixture of 4-aminomethylbenzoic acid and 2-IT in D_2O (p*D* ~7.5) at ambient temperature at several time intervals after mixing. The initial concentrations were: [4-aminomethylbenzoic acid] = 100 mM; [2-IT] = 76 mM. The broad water peak at δ 4.7 ppm has been partially shaded for clarity. The peak at δ 4.2 ppm is that of the methylene group of the remaining 4-aminomethylbenzoic acid (Ar-CH_2-NH_2).

prolonged incubation for 2 h also showed disappearance of the multiplet at δ 2.6 causing the peak at δ 2.2 to become a sharp triplet. These NMR data are consistent with the hypothesis that the secondary product is an *N*-substituted 2-iminothiolane.

Isolation and Characterization of N-Benzyl-2-Iminothiolane

The secondary non-thiol product from the reaction of benzylamine and 2-IT was isolated and purified. The results from elemental analysis, from mass spectrometric analysis, and from ^{1}H NMR analysis were consistent with the expected compound *N*-benzyl-2-iminothiolane (see Experimental). The ^{1}H NMR spectrum was recorded for a reaction mixture of benzylamine and 2-IT in D_2O at p*D* 7 that became turbid after 3 h; however, on acidification to p*D* 3.5, the reaction mixture became clear and its ^{1}H NMR spectrum was similar to that reported for 2-IT · HCl (1). The protonated *N*-benzyl-2-iminothiolane is therefore more soluble in water than its neutral form.

pK_a Values of 2-IT and N-Substituted 2-Iminothiolanes

The N-substituted 2-iminothiolanes showed remarkable stability in the presence of amines in comparison to 2-IT, and we reasoned that the contributing factor may be their lower pK_a in comparison to that of 2-IT. We determined, therefore, the pK_a values by taking advantage of the absorbance maxima at 248 nm of the protonated 2-iminothiolane moiety (1). The pK_a of 2-IT was measured as 7.6 based on the plot of absorbance at 248 nm vs pH (Fig. 4). The pK_a values of N-substituted 2-iminothiolanes derived from two representative amines, ethanolamine and glycinamide, were measured as 6.7 and 5.2, respectively.

Stability of N-Substituted 2-Iminothiolanes under Basic Conditions

The N-substituted 2-iminothiolanes generated by the reactions of 2-IT and amines of low pK_a (~8) were stable for several days at pH 8, even in the presence of excess amine, as observed by NMR and by their low thiol content (<2% of initial thiol). N-substituted 2-iminothiolanes generated from amines of high pK_a (~9.5) were somewhat less stable. While HPLC analysis showed that the N-substituted 2-iminothiolane generated by the reaction of ethanolamine (90 mM) and 2-IT (5 mM) was stable (<3% decay) on incubation for 3 h at ambient temperature at pH 8, 9, and 10, a purified sample of this N-substituted 2-iminothiolane upon a prolonged incubation for 2 days at pH 8 showed ~14% thiol generation. The amount of thiol generated from the purified sample was similar in the presence or ab-

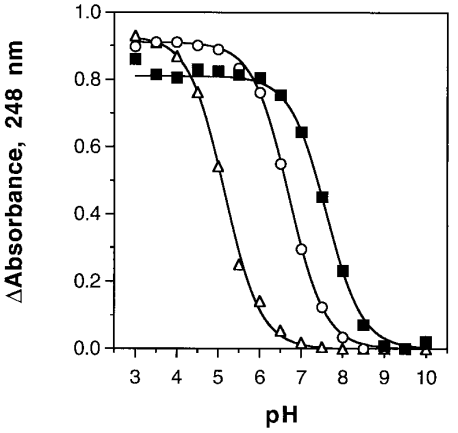


FIG. 4. Plots of ΔA at 248 nm vs pH for solutions of 2-IT (■) and N-substituted 2-iminothiolanes of glycinamide (△) and ethanolamine (○) in various 100 mM buffers. The N-substituted 2-iminothiolanes of glycinamide and ethanolamine were prepared by incubating 2-IT (5 mM) and the amine (20 mM) at pH 8 at ambient temperature for ~1 day; aliquots (30 μl) of the resulting solutions were diluted using 970 μl of buffer solutions, and the absorbance at 248 nm was measured in a cuvette of 1 cm pathlength. For 2-IT, aliquots of a 5 mM solution were diluted in a similar manner. The curves plotted are calculated curves which showed the best fit using the following pK_a values: 5.15 (N-substituted 2-iminothiolane of glycinamide), 6.7 (N-substituted 2-iminothiolane of ethanolamine), and 7.6 (2-IT).

sence of 50 mM ethanolamine, suggesting that the presence of excess ethanolamine does not affect the rate of generation of thiol from this N-substituted 2-iminothiolane.

Stability of 2-IT and N-Substituted 2-Iminothiolanes under Acidic Conditions

In a sample of an N-substituted 2-iminothiolane derived from the reaction of a glycopeptide and 2-IT and then purified by gel filtration at acidic pH, the formation of thiol was observed on prolonged incubation at pH 5. Solutions of several N-substituted 2-iminothiolanes were therefore acidified to pH 5, and the rates of generation of thiol were determined (Fig. 5). The N-substituted 2-iminothiolanes derived from amines of low pK_a (~8; Gly-Gly, glycinamide, Asp-Phe, Asp-Phe amide, Tyr-Gly-Gly, and asparaginamide) generated significant amounts of thiol over 5 days of incubation at pH 5 at ambient temperature, with the generation of thiol being nearly quantitative for the iminothiolanes of Asp-Phe, Asp-Phe amide, Gly-Gly, and glycinamide. In contrast, the iminothiolane derived from ethanolamine, an amine of high pK_a (9.5), did not generate a significant amount of thiol in solution at pH 5.

The rates of generation of thiol from such N-substituted 2-iminothiolanes and 2-IT were compared at pH 4, 5, and 6 in experiments analogous to that shown in Fig. 5. For 2-IT, the formation of thiol had the following trend: pH 6 > pH 5 > pH 4. For the N-substituted 2-iminothiolane of ethanolamine, the rates of thiol formation were approximately similar at pH 4, 5, and 6. For the N-substituted 2-iminothiolane of asparaginamide, the formation of thiol had the following trend: pH 4 > pH 5 > pH 6. For the N-substituted 2-iminothiolane of Gly-Gly, the trend was: pH 5 ≈ pH 4 > pH 6.

The chemical nature of thiol obtained upon acidification at pH 5 was analyzed by ^{1}H NMR spectroscopy and by the kinetics of reaction with *N*-ethylmaleimide (see below). The ^{1}H NMR spectrum of an acid-generated thiol sample derived from the reaction of Gly-Gly and 2-IT showed peaks at δ 2.5 and δ 1.9, which are characteristic peaks for the methylene groups α and γ (δ 2.4–2.6) to the thiol group and for the methylene group β (δ 1.9) to the thiol group in an *N*-substituted 4-mercaptobutyramide (R—NH—CO—CH_2—CH_2—CH_2—SH). The above ^{1}H NMR peak assignments are based on those of an authentic sample of the 4-mercaptobutyramide prepared by the reaction of Gly-Gly and γ-thiobutyrolactone. For amines of low pK_a, the acidification of N-substituted 2-iminothiolanes at pH 5 therefore results in hydrolysis and generates an N-substituted 4-mercaptobutyramide (R—NH—CO—CH_2—CH_2—CH_2—SH).

Stability of N-Substituted 2-Iminothiolanes to Hydroxylamine Treatment

The N-substituted 2-iminothiolanes derived from amines of high pK_a such as alanine and ethanolamine were treated with the strong nucleophile hydroxyl-

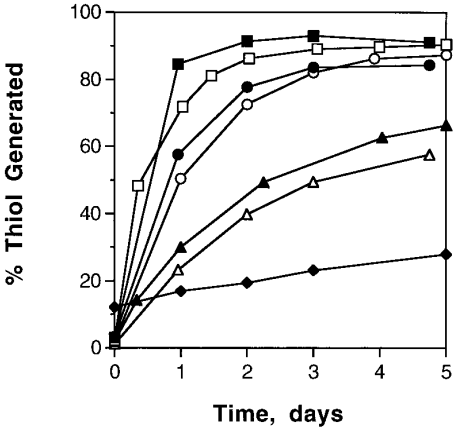


FIG. 5. Generation of thiol on acidification (pH 5) of non-thiol product (*N*-substituted 2-iminothiolane) from the reaction of 2-IT and amine [Asp-Phe (■), Asp-Phe amide (□), Gly-Gly (●), glycinamide (○), Tyr-Gly-Gly (▲) , asparaginamide (△) and ethanolamine (◆)]. The reaction mixtures of amines (22 mM) and 2-IT (5 mM) were incubated at pH 8 at ambient temperature for 1 day, and then were diluted (1:10) using 200 mM sodium acetate buffer, pH 5, containing EDTA (0.5 mM); the acidified solutions (pH 5) were incubated at ambient temperature and their thiol contents were measured using Ellman's assay at several time intervals. The maximum value (100%) of thiol is based on the amount of 2-IT used in the initial reaction.

amine. A sample of N-substituted 2-iminothiolane derived from alanine, when treated with 0.1 M hydroxylamine at pH 7.5 at ambient temperature, generated nearly quantitative amount of thiol within 1 min. The thiol formed, however, decayed rapidly with a half-life of 1 h. These results suggest the formation of *N*-hydroxy-2-iminothiolane with the release of alanine. In an experiment analogous to that shown in Fig. 3, this mechanism was confirmed by ^{1}H NMR analysis of a reaction mixture of the N-substituted 2-iminothiolane of 4-aminomethylbenzoic acid with hydroxylamine, which showed the release of 4-aminomethylbenzoic acid and the formation of a new N-substituted 2-iminothiolane (data not shown).

Kinetics of the Reactions of N-Ethylmaleimide with Thiols

Thiols of different pK_a values can be differentiated based on their reactivity with *N*-ethylmaleimide (NEM) (21, 22). We therefore thought that the 4-mercaptobutyramidines could be distinguished from the 4-mercaptobutyramides by the rate of reaction with NEM and so further confirm the identity of the thiol generated by acid treatment of N-substituted 2-iminothiolanes (Fig. 5). Rate constants (k_{app}; pH 5, 23°C) were compared for the reactions of NEM with several thiols generated in this study. The rate constants for the reactions of NEM with 4-mercaptobutyramidine (R—NH—C(=NH_2^+)—CH_2—CH_2—CH_2—SH) and 4-mercaptobutyramide (R—NH—CO—CH_2—CH_2—CH_2—SH) were 480 and 210 M^{-1} min^{-1}, respectively. The thiol group in R—NH—C(=NH_2^+)—CH_2—CH_2—CH_2—SH is therefore more reactive than that in R—NH—CO—CH_2—CH_2—CH_2—SH by a factor of 2.3. In a different experiment, the rate constant for the reaction of NEM with the thiol generated by acid hydrolysis of the *N*-substituted 2-iminothiolane of Gly-Gly (210 M^{-1} min^{-1}) was observed to be similar to that of R-NH-CO-CH_2-CH_2-CH_2-SH derived from the reaction of Gly-Gly and γ-thiobutyrolactone (220 M^{-1} min^{-1}). The thiol product generated by acidification of N-substituted 2-iminothiolane at pH 5 is therefore an N-substituted 4-mercaptobutyramide.

Substituent Effects on the Reactions of α-Amino Groups of Peptides with 2-IT

For the reactions of α-amino groups of peptides with 2-IT, the substituents at the α-carbon in peptides (especially nucleophiles and general acids or bases) may influence the formation and decay of the thiol adducts. We therefore studied the reactions of 2-IT with several peptides with different amino acids at the N-terminus, at pH 8 and 23°C (Fig. 6). Typically, a maximum value of thiol was reached within 20 min, showing a ΔA of about 1 unit in Ellman's assay (Fig. 6). The decay of the thiol was significantly faster for the thiol adduct of

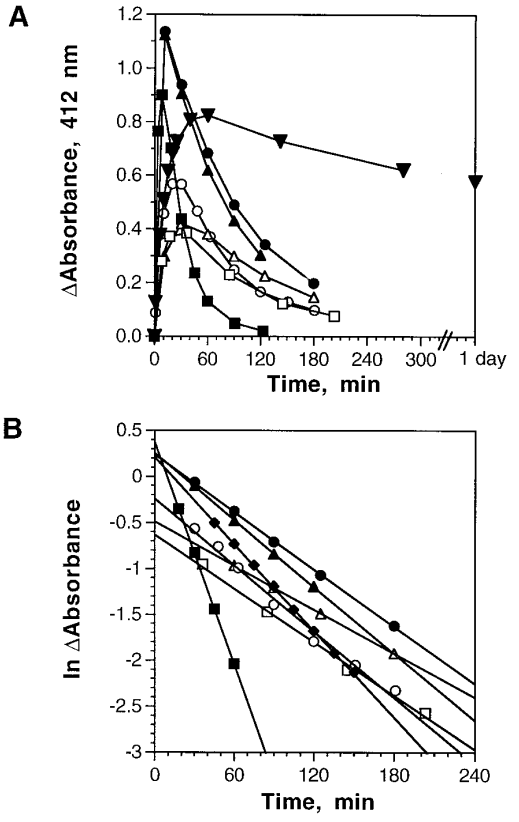


FIG. 6. Assay of thiol in reaction mixtures of several amines and 2-IT at 23°C. The initial concentrations were: [amine] = 22 mM, [2-IT] = 5 mM, [EDTA] = 5 mM; pH 8. Samples were taken at intervals and diluted 50-fold into Ellman's assay solution. (A) Plot of ΔA (Ellman's assay) vs time. (B) Plot of ln ΔA vs time, plotted for the time points after the maximum thiol had been generated (values for Thr-Val-Leu were not plotted in this graph). The values for Ala-Ala are from Fig. 1. [Gly-Gly (●); glycinamide (▲); Ala-Ala (♦); Asp-Phe (■); asparaginamide (△); Asn-Val (□); Tyr-Gly-Gly (○); Thr-Val-Leu (▼)].

Asp-Phe ($t_{1/2}$ = 0.3 h) than that for Gly-Gly ($t_{1/2}$ = 1.1 h). However, the linear first-order decay curves of ln ΔA vs time for both Asp-Phe and Gly-Gly, along with those of Ala-Ala and glycinamide, intersected at a value of ln ΔA of about 0.25 (~90% of the value expected for complete formation of thiol based on 2-IT) at Time 0–10 min (Fig. 6B).

In contrast, the maximum values of thiol generated for asparaginamide and Asn-Val were significantly lower than those for Gly-Gly and Asp-Phe, although the rates of decay of thiol ($t_{1/2}$ = 1.4–1.1 h) were similar to that for Gly-Gly (Fig. 6, Table 1). The linear decay curves of ln ΔA vs time for both asparaginamide and Asn-Val did not intersect at the common point of intersection observed for Gly-Gly, Asp-Phe, Ala-Ala, and glycinamide. Tyr-Gly-Gly behaved in a manner similar to the Asn-peptides, with a lower maximum value of thiol compared with the reaction of Gly-Gly, and a linear decay curve ($t_{1/2}$ = 0.8 h) that also did not intersect at the point common to Gly-, Asp-, or Ala-peptides (Fig. 6).

The reaction of Thr-Val-Leu with 2-IT showed the most surprising result that its thiol adduct was stable and showed little decay upon incubation at pH 8 for 1 day (Fig. 6A). The formation of this thiol adduct was not quantitative (about 60% of the value expected based on 2-IT) and also was slower than that for other peptides (Fig. 6A).

Decay of Thiol in a Sample of BSA Modified with 2-IT

2-IT is successfully used to introduce thiol groups into proteins and we therefore studied the fate of thiol groups introduced in BSA. BSA was first treated with iodoacetamide to alkylate its endogeneous thiol group. This sample was modified at pH 8 and 0°C with 2-IT, purified, and then shown by Ellman's assay to contain two thiol groups per BSA molecule. The modified BSA was then incubated at pH 7 at 25 and at 0°C and its thiol content monitored by Ellman's assay to establish a decay curve. The half-life of thiol introduced by 2-IT was determined from the curves as 2.7 h at 25°C and 44 h at 0°C. The modified BSA samples after 2 weeks at 0°C showed an absorbance maxima at 250 nm at pH 3.2, that decreased significantly at pH 7, therefore indicating the formation of an N-substituted 2-iminothiolane moiety.

For a control sample of BSA where ~7 thiols had been introduced by the reactions with SPDP and DTT, the decay of thiol at pH 7 was less than 4% in 3 h at 25°C, and less than 4% in 44 h at 0°C. The thiol groups introduced in BSA using 2-IT are therefore less stable than those introduced using SPDP and DTT. The half-life of the thiol groups introduced in BSA using 2-IT is similar to that observed for the initial thiol adduct of ethanolamine, as expected because the pK_a of the amino group in ethanolamine (9.5) is similar to that of the ϵ-amino groups in lysine residues in proteins (pK_a about 10).

DISCUSSION

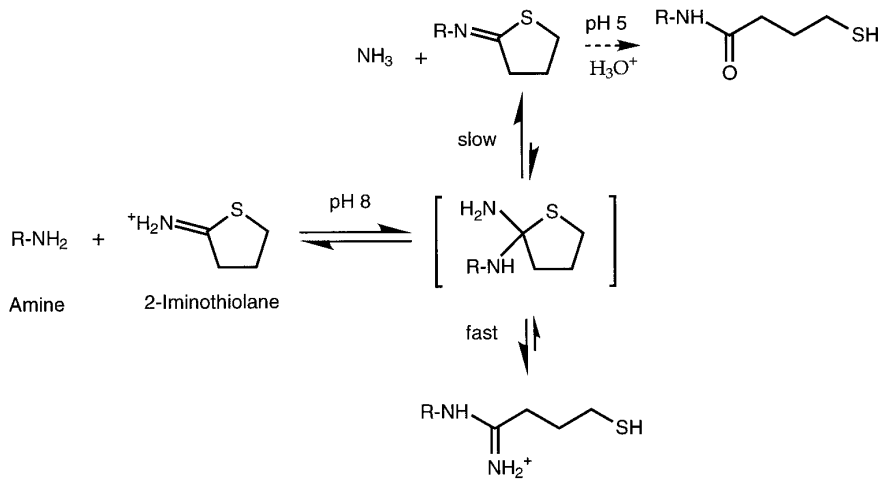
The reaction of an amine with 2-IT is fast at pH 8 and results initially in the formation of a thiol adduct. This initial thiol adduct, however, is unstable and decays by first-order kinetics to a non-thiol product. The half-lives of the initial thiol adducts vary with the amine, ranging from 0.3 to 3 h at 23°C. The decay of the initial thiol adduct is much slower at 0°C, with half-lives ranging from 1 to 44 h. This decay of the initial thiol adduct of an amine and 2-IT is not affected by the presence of EDTA or DTT, and is, therefore, not due to its oxidation to a disulfide.

A scheme is proposed below for the reaction of amine with 2-iminothiolane, in which the initial tetrahedral intermediate can break down by two pathways: (i) by the rapid formation of a thiol with an amidine functionality; (ii) by the slow formation of an N-substituted 2-iminothiolane (non-thiol product) with the release of ammonia (Scheme 2). The two pathways are in equilibrium with the tetrahedral intermediate. Although the formation of thiol is more rapid, the overall equilibrium is shifted toward the formation of the N-substituted 2-iminothiolane (non-thiol product). The reaction therefore results in the initial formation of thiol followed by a gradual disappearance of thiol and formation of a non-thiol product. The thiol formed initially can be trapped by lowering the pH to ~3–4, or by its modification to a disulfide.

The N-substituted 2-iminothiolane product is stable, even in the presence of excess amine in the reaction mixture. The low reactivity of N-substituted 2-iminothiolane, compared to that of 2-IT, is presumably a combined effect of the low pK_a of its protonated form and the steric effect of the N-substituent. The pK_a values of N-substituted 2-iminothiolanes derived from the reactions of 2-IT with ethanolamine and glycinamide are 6.7 and 5.2, respectively, and are significantly lower than that of 2-IT (7.6). The reactive species in the reaction of an amine with 2-iminothiolane are the unprotonated amine and the protonated 2-iminothiolane. Amines react with 2-IT at pH 8 because a significant proportion of both reactive species are present. In contrast, for the reaction of an N-substituted 2-iminothiolane with amine at pH 8, there is not a significant proportion of the protonated N-substituted 2-iminothiolane present. In addition, the steric effect of the substituent in N-substituted 2-iminothiolane may lower its reactivity. N-substituted 2-iminothiolanes are stable to amines, in contrast to N-substituted imidates that are formed in the reactions of amines with imido esters at pH 8 and which can then further react with amines to give disubstituted amidines (23).

The kinetics of formation of thiol adducts in the reactions of asparaginamide, Asn-Val and Tyr-Gly-Gly with 2-IT are anomalous, and may involve the participation of the side-chain amide or hydroxyl residues. The initial thiol adduct of 2-IT with Thr-Val-Leu is also anomalous, being much more stable than the initial thiol adducts of the other peptides tested, perhaps due to the formation of a substituted 2-(3-mercaptopropyl)oxazoline by the reaction of the hydroxyl group in threonine. The rapid decay of the initial thiol adduct of Asp-Phe is presumably due to general acid catalysis by the side-chain carboxyl group in Asp.

The N-substituted 2-iminothiolane products derived from amines of low pK_a values (~8; e.g., Gly-Gly, Ala-Ala) hydrolyze slowly upon acidification at pH 5 to generate N-substituted 4-mercaptobutyramides (R-NH-CO-CH_2-CH_2-CH_2-SH). Compounds with similar functional groups such as substituted thioimidate esters and thiazolines have been reported to hydrolyze under acidic conditions at pH 5.0 resulting mainly in the formation of thiol compounds with amide moieties due to C—S cleavage, although a second pathway with



SCHEME 2

C-N cleavage can be significant at pH 2–3, resulting in the formation of a thioester and amine (24–26). While the initial thiol adducts formed by the reaction of α-amino groups of peptides with 2-IT are inherently unstable even at 0°C, acidic hydrolysis of the N-substituted 2-iminothiolane secondary product at pH 5 allows the generation of a stable thiol adduct, the N-substituted 4-mercaptobutyramide. Awareness of these secondary reactions suggests that it is likely that the glycopeptide ligand described previously for modification of the galactose-binding sites of ricin has an amide functionality rather than an amidine (15).

When using 2-IT to introduce thiols into peptides or proteins for use in coupling or cross-linking reactions, it is important to be aware that the initial products of such reactions, N-substituted 4-mercaptobutyramidines, undergo secondary reactions. If the thiol adduct itself is simply an intermediate for a subsequent reaction, such as a disulfide cross-linking reaction (3–5) or in conjugate formation (12, 13), it is essential to minimize the degeneration of the thiol by maintaining low temperature and proceeding rapidly. Preferably, a thiol-modifying agent is present in excess in the initial reaction mixture of amine and 2-IT so that the 4-mercaptobutyramidine can be capped *in situ*. For instance, when 2-IT is used for disulfide cross-linking, it is preferable to stabilize the initial thiol adduct by its immediate reaction with DTNB or 2,2′-dithiodipyridine, generating a reactive mixed disulfide that can be used for subsequent coupling to a thiol-containing molecule (13, 14). However, it should be realized that upon reversal of such a disulfide cross-link, the N-substituted 4-mercaptobutyramidine will decay to an N-substituted 2-iminothiolane resulting in loss of thiol, lowering of the pK_a, and gain of a chromophore at 248 nm.

In cases where the mercaptoamidine is the desired final product, use of the reagent methyl 3-mercaptopropionimidate (27) is expected to yield stable thiol adducts which are not likely to cyclize to the unfavorable four-membered ring, thus retaining the positively charged amidine functionality. In cases where the use of the longer 2-IT reagent is appropriate for cross-linking, one further consequence of the eventual formation of an N-substituted 2-iminothiolane is that the original amine can be regenerated under mild conditions upon incubation with hydroxylamine (0.1 M) at pH 7.5. Awareness that the 2-IT modification can be easily reversed under these mild conditions further increases the practical value of 2-IT as a reagent for modifying amines in proteins and peptides.

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