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Thiol detection, derivatization and tagging at micromole to nanomole levels using propiolates

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Abstract

Thiols, simple and complex, including polypeptide and protein thiols, react rapidly and selectively with esters of propiolic acid under very mild conditions (aqueous buffer, room temperature, pH 7) to give thioacrylates. These stable derivatives exhibit strong, characteristic ultraviolet spectra, maximal at 280–290 nm, molar absorbance *ca* 12,500. In a single, simple experiment the thiol is detected, its amount is estimated, it is stabilized against oxidation and disulfide scrambling, it is converted into a derivative amenable to isolation and structure elucidation procedures, and it is tagged with recognizable ultraviolet and NMR characteristics generated *de novo*. The reagents are stable and inexpensive and the procedures are quick, easy, sensitive and selective.

Keywords: Thiol detection; Thiol derivatization; Sulfhydryl protein; Propiolate; Thioacrylate

1. Introduction

The purpose of this paper is to bring to attention the reaction of thiols with simple esters of propiolic acid as a versatile and potentially powerful device for their investigation. Thiols (mercaptans, sulfhydryl compounds) are widespread and significant in many areas of chemistry, biology and biochemistry [1,2]. Many reagents and procedures have been devised for their investigation [3–8]. Some of these detect and estimate the amount of thiol; some stabilize it against oxidation and disulfide scrambling and convert it into a stable derivative which can be isolated and subjected to structure elucidation procedures; yet others attach a chromophore or fluorophore, a trackable tag, to the sulfur atom. This paper describes reagents and procedures which do all of these things in one simple experiment.

2. Materials and methods

2.1. Materials

Ethyl and methyl propiolates (EP, MP)[1] were used as received, no purification needed. These alkynes are remarkably stable. EP from a bottle which had been kept at room temperature under ordinary laboratory conditions (brown bottle) for 35 years (*sic*) gave results indistinguishable from those obtained with fresh material. EP is soluble in water to 10–12 mg/mL (~0.1 M). MP is more soluble (0.4 M). Both esters are more soluble in 20% ethanol and in 8 M urea. EP, MP, tris-carboxyethylphosphine HCl (TCEP·HCl), thiols, amino-acids, buffers, urea, coenzyme A, insulin, glyceraldehyde phosphate dehydrogenase (GPD) and other materials were from Sigma–Aldrich.

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[1] *Abbreviations:* EP, ethyl propiolate; MP, methyl propiolate; DTT, dithiothreitol; TCEP, tris-carboxyethylphosphine; GPD, glyceraldehyde phosphate dehydrogenase; *cev*, carbethoxyvinyl; *cmv*, carbomethoxyvinyl.

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2.2. Instruments

UV: Agilent 8453 UV–vis. NMR: Varian Inova 400. GC–MS: Shimadzu GCMS-QP5000. LC–MS: Agilent Technologies LC/MSD VL.

2.3. General procedures

2.3.1. Detection and estimation

Buffers are phosphate, 2, 5, 10 and 50 mM, pH usually 6.8 or 7.5. Propiolate ester concentrations need not be accurately measured and stock solutions are not used. Into 3 mL of buffer in a 1 cm cuvet is introduced one small droplet (~12 mg) of EP from a Pasteur pipet to give a 40 mM concentration, 2 droplets for 80 mM, one droplet of a 50% mix or a 25% mix with ethanol to give 20 or 10 mM. The cuvet is upended 15–20 times (or more) to ensure uniform dissolution, and the instrument is blanked on the solution. Analyte, 50–200 μL, 50–200 nmol, is added, the cuvet is quickly upended three or four times more, and the absorbance at the maximum (or at chosen wavelengths) is observed and monitored until no further increase occurs.

2.3.2. Recovery and characterization

Excess reagent may be removed from the 3 mL of UV solution by sparging with inert gas or by pumping on the rotary evaporator. Solvophilic thiol derivatives are extracted into dichloromethane (0.5 mL), the solutions introduced into the GC–MS, and the electron-impact fragmentation patterns determined. The carbethoxyvinyl (*cev*) derivatives of dithiothreitol (DTT), methyl thioglycollate, mercaptoethanol, acetylcysteamine, mercaptopropionic acid and tiopronin thus were confirmed. For hydrophilic thioacrylates the aqueous solution is lyophilized, the residue extracted with methanol, methanol– acetonitrile, or methanol–acetonitrile–water mixture, and the extract introduced into the LC–MS. Electrospray mass spectra thus obtained confirmed the MH^+ masses for *cev* cysteine, glutathione and homocysteine. For NMR without chromatography, derivatives are extracted into appropriate deuterated solvents ($CDCl_3$, CD_3CN, etc.).

2.4. Specific procedures

2.4.1. Coenzyme A

A 1.5 mg (approx) sample was dissolved in 38.0 mL of buffer (2 mM pH 6.8). The solution had λ max 260 nm, A max 0.825, consistent with 1.46 mg of coenzyme. The spectrophotometer was blanked on 3.0 mL of the solution and one droplet of 50% EP was mixed in. Absorbance developed with λ max 285 nm, and a plateau value of 0.635 was reached within 30 min. Corrected for propiolate absorbance (0.032) this gives a thioacrylate:adenylate absorbance ratio of 0.73:1, close to the expected 0.75. Repeat determinations 0.71 and 0.74.

2.4.2. Insulin

A mixture was made of urea (90 mg), 50 mM sodium dihydrogen phosphate (70 μL), 50 mM disodium hydrogen phosphate (65 μL), insulin solution (Sigma, nominal 10 mg/mL, 110 μL) and 0.1 M TCEP·HCl (30 μL). The solution was kept under nitrogen overnight and diluted to 10.0 mL with 8 M urea. Observed absorbance *vs* 8 M urea was 0.082 at 280 nm. The spectrophotometer was blanked on this solution, one droplet of EP was mixed in, and absorbance at the maximum, 283 nm, was monitored for 120 min. The fast thiol reaction was complete within 5 min. ($A = 1.284$) after which a slow linear rise continued. A simple deconvolution was effected by drawing a line through the origin paralleling the after-reaction and taking as the thioacrylate absorbance the interval between the two lines. Corrected for EP (0.088) and TCEP (0.012) the observed thioacrylate:tyrosine ratio was 14.1:1. Repeat determinations gave 13.5 and 14.3, average 14.0, calculated 14.6.

2.4.3. GPD

Crystalline lyophilized enzyme (Sigma, 2 mg) was dissolved in 3.0 mL of 2 mM-phosphate-buffered 8 M urea pH 6.8. The solution had $A = 0.458$ at λ max 276 nm. Blanking and addition of EP (1 droplet) generated a fast initial reaction followed by a slow after-reaction just as with reduced insulin. Deconvolution and correction for EP gave a thioacrylate:protein absorbance ratio 0.42:1, repeats, 0.40 and 0.44. Another sample, reduced with TCEP as described for insulin, gave a ratio of 2.15:1.

2.4.4. Typical synthesis, preparative scale

cis-Ethyl 3-(2′-Amino-2′-carboxyethylthio)acrylate (*cev* cysteine). To cysteine (121 mg, 1 mmol) in water (2 mL) was added EP (100 mg, 1.02 mmol) in ethanol (0.5 mL) and 1 drop of 50 mM disodium hydrogen phosphate solution. Crystallization of product commenced within 30 s. Recrystallized from water, the pure *cis* isomer (140 mg, 65%) had mp 202–203°C.; NMR (D2O) δ 1.21 (t, 3H), 3.30 (m, 2H), 3.96 (t, 1H), 4.15 (q, 2H), 5.94 (d, 1H, $J = 10.2$ Hz), 7.26 (d, 1H, $J = 10.2$ Hz); MS (electrospray), MH^+ 220, 13-C MH^+ 221 (11%), 34-S MH^+ 222.1 (5%); UV (H2O) λ max 280 nm, ε max 12,400.

3. Results and discussion

3.1. Simple thiols

That thiols react with propiolate esters to give thioacrylates has been long known [9,10].

$$R{-}SH + HC{\equiv}C{-}COOR' \rightarrow R{-}\ S{-}CH{=}CH{-}COOR'$$

What has not been appreciated is the remarkable speed of the reaction and the very mild conditions it requires . What also has been known for a long time but has not been exploited for investigational purposes is the very substantial ultraviolet spectrum of the resulting thioacrylate, as

contrasted with the virtual transparency [11] of the precursor propiolate. Thus, in a typical experiment, when a 200 nmol sample of cysteine was dispersed into a solution of 25 mg of EP in 3 mL of buffer, pH 6.8, a most prominent UV absorbance, λ max 280 nm, $A > 0.5$, developed as quickly as the spectrophotometer could be read. Half-life for the pseudo-first-order reaction was about 12 s, and within 2 min a plateau value was reached, $A = 0.79$, which translates to a molar absorbance of ~12,500. Smaller and larger quantities of thiol (50–500 nmol) gave comparable results. With precautions to preclude adventitious oxidation even smaller amounts should be detectable. The reaction rate can be manipulated widely by adjusting propiolate concentration and pH. Lowering the pH to 5.8 slowed the rate 10-fold, raising it to 7.8 accelerated it similarly. Lowering propiolate from 80 mM to 20 mM gave a one-minute half-life at pH 6.8 (convenient for kinetic analysis) while 400 mM, attainable with MP or with 20% ethanol as co-solvent, gave complete reaction within 25 s. In the extreme case, pH 8 and 400 mM ester, complete reaction would be achieved within a second or two, which is likely to be useful in systems requiring rapid blocking against disulfide scrambling [7a,12]. With all the model compounds examined (Table 1) detection was quick and easy, and molar absorbance values of 12,500 ± 5–10% were the rule.

3.2. Structure–activity correlations

Table 1 lists the simple thiols chosen for model study. Cysteine is the reference point. Glutathione models cysteine peptides. DTT is a dithiol. Cysteamine, *N*-acetyl cysteamine, and other items 4–10 have less crowding about the sulfhydryl group than does cysteine, while tiopronin has more. Methyl thioglycollate is a particularly acidic thiol while mercaptopropionate is much less acidic, comparable to simple alkanethiols (butanethiol pK_a 10.6). Coenzyme A has the same stereoelectronic environment about the sulfhydryl group as the much smaller acetylcysteamine.

Two useful structure–activity relationships may be gleaned from the data in Table 1. First, reaction rate for structurally comparable thiols relates directly to the acidity of the thiol group. Second, steric crowding, resulting from branching at the carbon centers β or α to the thiol group, engenders a modest and predictable rate diminution. Reaction proceeds through the thiolate ion, of course, and the concentration of this is governed directly by the acidity constant of the thiol. Methyl thioglycollate is the most reactive and mercaptopropionate is the least reactive of the thiols listed. Multiplying the half-life by the acidity constant of the thiol gives the relative half-life for reaction of the thiolate ion, the reciprocal of which is the relative rate constant for that ion. These rate constants, normalized on cysteine as unity, are shown in the right-hand column of the Table. The constants for items 5–10 show that the structurally diverse ions unbranched on the carbon atoms α and β to the thiol have very comparable reactivities. Items 1–3 show that branching at the carbon atom β to the thiol group slows reaction of the thiolate ion by 2 to 3-fold. Item 11 shows the α-branched tiopronin to be five times less reactive still. It is noteworthy that the size and complexity of the branch or tail have little effect. Glutathione is much less acidic than cysteine and the branches are much bulkier, yet the thiolate ion reactivities are, within experimental error, identical. The coenzyme A ion is just as reactive as the simpler unbranched structures. These correlations may be of significant predictive and diagnostic usefulness.

Table 1
Reaction of thiols with 80 mM ethyl propiolate in phosphate buffer, pH 6.8

	Thiol	Half-life	pK_a	k (RS–) (Rel)[a]
1	Cysteine	12 s	8.3	1
2	Glutathione	30 s	8.7	1
3	DTT	2 min	9.3	1
4	Methyl thioglycollate	2 s	7.8	~1.9
5	Cysteamine	10 s	8.6	2.4
6	Homocysteine	22 s	8.9	2.2
7	Mercaptoethanol	75 s	9.5	2.5
8	*N*-Acetyl cysteamine	80 s	9.5	2.3
9	Coenzyme A	100 s	9.6	2.4
10	Beta-mercaptopropionic acid	7 min	10.3	2.8
11	Tiopronin	20 s	7.8	0.2

Thiol half-lives and thiolate-ion relative pseudo-first-order rate constants.
[a] Normalized on cysteine = 1. Accuracy about 10%.

3.3. Stereochemistry

Thioacrylates exist as *cis* and *trans* stereoisomers. Both are produced when thiols react with propiolates, but previous studies [10] show that under the conditions used in the present work the product is likely to be 85–95% *cis*. The *trans* isomers have their UV maxima at lower wavelength and absorb more strongly than the *cis*, but the absorbance curves cross, so that the isomers have about the same absorbance as each other at the maximum of the *cis* isomer [13,14]. Thus, within the limits of accuracy of the method, the observed maximal absorbance can be treated as though it were all due to *cis*, even though the position of the maximum is shifted to slightly lower wavelength.

3.4. Quantity estimation

All thioacrylates are different substances and each has its own molar absorbance, but values between 11,500 and 13,500 seem to be typical [10,13,14]. Using an average of ~12,500, the plateau value reached in the detection of an unknown thiol provides a useful estimate of its amount to a level of accuracy of ±5–10%, sufficient for most purposes.

3.5. Coenzyme A

Coenzyme A is self-referencing . The adenylate chromophore in the backbone generates a maximum at 260 nm, molar absorbance 16,800. Absent other chromophores,

Table 2
Reaction of mercaptopropionate and interfering nucleophiles with 40 mM ethyl propiolate in phosphate buffer, pH 7.5

	Time (min)	2	4	8	12	16	20	40	80	160	400
Molar absorbance											
1.	Mercaptopropionate	4600	7300	10,200	11,250	11,900	12,100				
2.	Proline	150	250	425	600	750	850	1500	2050		3800
3.	Pyrrolidine			400	500	550	625	950	1450		2600
4.	Guanylate			250	350	400	450	600	750	900	
5.	Uridylate			90	125	150	180	300	450		
6.	Tyrosine			80	110	140	175	250	350		
7.	Histidine			80	110	140	170	250	350		
8.	Lysine			70	100	120	140	250	375		
9.	TCEP	30	40						60		
10.	Urea							0.02		0.04	

Development of absorbance, per mole, with time.

the absorbance of the sample tells how much of the coenzyme we have. Since the thiol generates a thioacrylate molar absorbance of about 12,500, if the analyte solution is measured at 260 nm, blanked, reacted with propiolate, and remeasured at the maximum (285), the ratio of the two absorbances should be 12,500:16,800 or 0.75:1. In typical analyses a ratio of 0.73 ± 0.2 was found. The ratio fell to 0.62, indicating 15% oxidation to disulfide, when the thiol solution was kept in air overnight.

3.6. Interfering nucleophiles

Many nucleophiles react with propiolates to give adducts which exhibit intense UV absorbance [9,15,16] . If propiolates are to be of use in the manipulation of thiols it is necessary to know how such competitors might interfere. The behavior of a selection of the kinds of nucleophiles likely to be encountered in biological systems is recorded in Table 2, along with the behavior of mercaptopropionate, by far the slowest-reacting of the thiols in Table 1. It is apparent that not one of the competitors generates optical density anywhere near as fast as even this least reactive thiol does. In the time that it takes mercaptopropionate to generate 93% of its eventual absorbance (12 min, 4 half-lives) even proline generates barely 5%, pyrrolidine a little less, guanylate, typical of the most reactive purines and pyrimidines, 3%, and uridylate barely 1% of the absorbance that the thiol generates. Tyrosine, histidine and lysine are hardly at all more reactive than the common monoamino-monocarboxylic acids. Even with an unreactive thiol, then, it would require an unusual situation for detection to be obscured, estimation precluded, or derivatization interfered with TCEP is included in the table. It is a reagent of choice for the reduction of disulfides [17] especially cystine peptides [7a,12], and an excess of it invariably is present in the resulting thiols. TCEP reacts rapidly with propiolates, but fortunately the reaction generates little absorbance.

3.7. Derivative isolation, tracking and characterization

Thioacrylates, the carbethoxyvinyl (*cev*) and carbomethoxyvinyl (*cmv*) derivatives of the thiols, are stable to oxidation and to disulfide scrambling. Authentic specimens are easily prepared. The *cev* derivative of mercaptopropionic acid is a known compound [10] All the *cev* derivatives and most of the *cmv* derivatives of the thiols listed in Table 1 have been prepared and characterized. This synthetic work will be reported elsewhere. A typical example, *cev* cysteine, is described in Section 2. Current synthetic work targets derivatives of small cysteine peptides. One useful feature of propiolates as derivatizing agents is that they can be used in large excess, ensuring rapid and complete reaction, and the excess can then be removed simply by evaporation. A major feature, of course, is the UV absorbance of the thioacrylate group generated *de novo*, which will permit easy recognition in chromatographic eluates. A third feature, even more incisively characteristic than UV, is the NMR spectrum. The protons of the vinyl group appear as a very widely separated doublet of doublets, chemical shifts *ca* 5.9 and 7.2 δ for the *cis* isomer, coupling constant *ca*10 Hz. These resonances occupy what is usually a relatively empty NMR region. Even in a spectrum as complex as that of *cev* coenzyme A they are easily identified. NMR requires little more sample than UV. Procedures developed here for model systems should be readily applicable to unknown thiol-containing materials. Easy removal of excess derivatizing agent, tracking of the newly generated UV-absorbing entities in conventional or reversed-phase chromatographic eluates, mass spectra, NMR, and if needed, 2-dimensional NMR spectra, will yield penetrating structural information. Some cell extracts and biological fluids are opaque at 280 nm. Propiolates cannot detect thiols in them, but they still can be used to produce thioacrylate derivatives which can be tracked, isolated and characterized.

3.8. Proteins

It would seem that propiolates should have considerable potential to complement other derivatizing agents such as iodoacetamide, iodoacetate, vinyl pyridine, acrylates and maleimides in investigations of cysteine- and cystine-containing proteins. Some model work to probe this potential has been done with two proteins, insulin and glyceralde-

hyde phosphate dehydrogenase. GPD (rabbit) is a tetramer. The native, freshly isolated, active enzyme has four thiol groups in each of the four primary chains. It is extremely susceptible to oxidation, however, with concomitant loss of enzymatic activity [18], and the ordinary commercial material, as received, has an uncertain thiol content. This makes it ideal for the present purpose, which is not to do enzymology but rather to determine whether and how propiolate might be able to detect, estimate and fix an indeterminate number of thiol groups in a big protein. GPD has three tryptophane residues and nine tyrosines in each primary chain, and so, like coenzyme A is self-referencing; as, indeed, most proteins will be. Standard values for trp and tyr [7b] require the molar absorbance of the unfolded protein (8 M urea) at the maximum (282 nm) to be 27,600. Reaction of undenatured protein proved to be slow. In 8 M urea, however, development of absorbance at the maximum, 283 nm, was just about as fast as with glutathione, and the final value, suitably corrected, corresponded to a thioacrylate:backbone absorbance ratio of 0.42 ± 0.02. One thiol group would require 12,500: 27,600, or 0.45. A slow but distinct after-reaction was observed, the absorbance at 283 nm rising an additional 20% between 5 min and 60 min and continuing to rise thereafter. Nevertheless, the thiol reaction was comfortably fast enough to be easily deconvoluted from the after-reaction. GPD reduced with TCEP gave an absorbance ratio of 2.15, 20% high for four cysteines.

Insulin, also in 8 M urea, was reduced with TCEP much as described by Gray [7a,12]. Insulin has three disulfides, four tyrosines and no tryptophane residues, which mandates an absorbance of 5120 for the reduced protein and a 14.6:1 thioacrylate: backbone ratio after carbethoxyvinylation. Results in good agreement with this ratio were obtained in multiple experiments. Half-life for reaction with thioacrylate once again was comparable to glutathione, all six thiol groups seeming to react at the same rate. Once again an after-reaction was seen, absorbance at the maximum again rising some 20% between 5 and 60 min. One experiment was stopped at 5 min and the protein content examined by SDS–PAGE. Two bands were observed, clearly corresponding to *bis-cev* B-chain and *tetrakis-cev* A-chain. Gray [12] in his elegant studies unraveling the structures of "knotty" disulfide cross-linked proteins used a supersaturated solution of iodoacetamide (2.2 M) to trap partially reduced proteins while avoiding disulfide scrambling as much as possible at the elevated pH required by the carboxamidomethylation reaction. The findings reported here show that propiolates react at least as fast as iodoacetamide, at lower pH, can be used in very high concentrations, and give *cev* and *cmv* derivatives in a manner that may be of use in such work.

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