

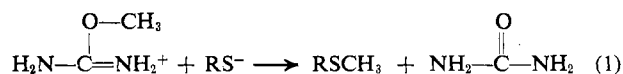
S Methylation of Thiols by *O*-Methylisourea*

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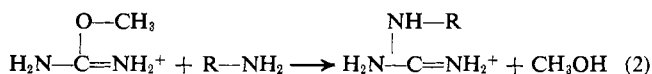
ABSTRACT: Reactions between cysteine and *O*-methylisourea were studied as a function of pD in D₂O at 28° using a nuclear magnetic resonance spectrometer to follow the reactions. Signals from the methyl protons on the methyl groups of *O*-methylisourea, methanol, and *S*-methylcysteine could be clearly distinguished from each other, so that competition between α guanidination and S methylation of cysteine could be observed. The pH dependence of the rate of S methylation of 2-mercaptoethanol by *O*-methylisourea was also studied. The apparent second-order rate constants for S methylation are proportional to the product of the fraction of thiol anion and *O*-methylisouronium cation present at the pD or pH at which the reaction is run. The second-order rate constant for the attack of the thiol anion of 2-mercaptoethanol on *O*-methylisouronium cation is $1.1 \times 10^{-2} \text{ min}^{-1} \text{ M}^{-1}$ at 28° in water. No reaction

other than guanidination of an amino group could be detected from nuclear magnetic resonance spectra, when *O*-methylisourea was incubated for 24 hr at pD 8.9 with arginine, asparagine, aspartic acid, glutamine, glutamic acid, histidine, methionine, threonine, or serine. Imidazole (1 M), ethanol (1 M), or acetate (1 M) failed to react (as judged from the constancy of the nuclear magnetic resonance spectra) with *O*-methylisourea (initial concentration 0.1 M) after 24 hr at pD 8.9. These results strongly suggest that *O*-methylisourea could be a useful reagent for selectively alkylating thiol groups in proteins. The occurrence of S methylation at appreciable rates at pD 10–12 suggests that conditions normally used to guanidinate a protein (*i.e.*, incubation of a protein for long times with concentrated solutions of *O*-methylisourea) could lead to substantial S methylation of thiol groups present in a protein.

In a previous study, we observed that an isoureyline derivative of a seryl residue at the active site of α -chymotrypsin reacted with 2-mercaptoethylamine to yield an *S*-aminoethylcysteinyl residue (Banks *et al.*, 1969). This observation led us to investigate the feasibility of using isoureas, such as *O*-methylisourea, as specific alkylating agents for thiol groups in proteins (eq 1).



Although *O*-methylisourea has been used extensively for over 20 years to guanidinate primary amino groups in proteins (eq 2), reports of S methylation of thiol groups by *O*-methylisourea appear to be absent from the literature. (The use of *O*-methylisourea as a guanidinating agent has been reviewed by Kimmel, 1967.)



In this work, reactions of *O*-methylisourea with cysteine, 2-mercaptoethanol, and several amino acids are studied to

obtain information regarding the relative reactivities of thiol and amino groups toward *O*-methylisourea.

Materials and Methods

Amino acids were A grade from Calbiochem. *O*-Methylisourea hydrogen sulfate was obtained from Aldrich. 5,5'-Dithiobis(2-nitrobenzoic acid) was obtained from Sigma. Imidazole from Aldrich was recrystallized from benzene three times and resublimed. Deuterium oxide, 99.8%, was from Bio-Rad. 2-Mercaptoethanol was from Eastman Organic Chemicals. The distilled water supplied to the laboratory was run through a demineralizer and redistilled in an all-glass still. All other chemicals used were Fisher, Mallinckrodt, or Baker Adamson analytical reagents.

Measurements of pD were made using a Radiometer Model 4b pH meter or a Radiometer TTT-lc pH-Stat, which was standardized with a 1:1 phosphate-NBS primary standard solution (Bates, 1964). The response of the glass electrode was checked with another NBS primary standard solution (borax). Any nonideality in the glass electrode response was corrected with the temperature compensator. The pD was obtained using the relationship, pD = glass electrode reading + 0.40 (Bates, 1964).

Rate Measurements. Solutions of *O*-methylisourea and amino acid were mixed and quickly brought to the desired volume and pD in a jacketed beaker, which was maintained at $28 \pm 0.1^\circ$ by circulating water from a Lauda U3 water bath. Reactions were usually run in 10^{-3} M EDTA solutions, which were previously degassed to minimize oxidation of cysteine. (Running the reaction under a stream of nitrogen leads to loss of methanol.) When necessary, the pD of the reacting solution was kept constant using a Radiometer

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TABLE I: Rate Constants for S Methylation and Guanidination of L-Cysteine by O-Methylisourea.^a

pD	10 ³ k _M ^b (min ⁻¹ M ⁻¹)		10 ³ k _{MEOH} ^c (min ⁻¹ M ⁻¹)	10 ³ k _G ^d (min ⁻¹ M ⁻¹)
	Obsd	Calcd ^e		Obsd
7.37	0.64	0.68		
8.38	3.6	4.2		
8.87	8.0	6.8	0.81	0.80
9.36	8.6	8.4	2.1	2.0
9.95	8.8	8.4	7.9	7.7
10.39	6.3	7.1	13.3	13.1
10.91	4.5	4.3	13.9	13.7
11.46	1.6	1.7	6.8	6.7

^a At 28°, 10⁻³ M EDTA. Initial concentration of cysteine, 1 M; initial concentration of O-methylisourea, 0.1 M. The estimated accuracy of rate constants is ±10%. ^b k_M represents the apparent second-order rate constant for S methylation. ^c k_{MEOH} represents the apparent second-order rate constant for formation of methanol. ^d k_G, the apparent second-order rate constant for guanidination, was determined by subtracting the rate constant for formation of methanol in the absence of cysteine from k_{MEOH}. ^e Calculated from the relationship

$$k_M = \frac{\left(k_{MB}^0 + \frac{k_{MC}^0 k_{123}}{[\alpha_D]} \right) k_{12}}{\left(1 + \frac{K_2}{[\alpha_D]} + \frac{K_2 K_3}{[\alpha_D]^2} \right) ([\alpha_D] + K_1)}$$

with k_{MB}⁰ = 1.3 × 10⁻² min⁻¹ M⁻¹ and k_{MC}⁰ = 2.4 × 10⁻² min⁻¹ M⁻¹. The apparent dissociation constants, k₁₂₃ = 2.4 × 10⁻¹¹, k₁₂ = 2.4 × 10⁻⁹, K₂ = 3.2 × 10⁻⁹, K₃ = 1.8 × 10⁻¹¹, and K₁ = 4.0 × 10⁻¹¹ were determined by titration (see text).

TTT-lc pH-Stat coupled to an Aminco Automatic buret. In many runs, however, the buffering capacity of the reaction mixture was sufficient for maintenance of constant pD within 0.04 pD unit, so that use of the pD-Stat was unnecessary.

For reactions of O-methylisourea (initial concentration 0.1 M) with cysteine (initial concentration 1.0 M), the time dependencies of the concentrations of methanol, O-methylisourea, and S-methylcysteine were obtained from nuclear magnetic resonance spectra (using a Varian T-60 spectrometer) by integrating the signals from the methyl protons in these compounds.

When the reaction was run at pD 7.4, or above pD 9.9, the pD of the reaction mixture had to be adjusted to approximately pD 8.5 prior to obtaining the nuclear magnetic resonance spectra, in order to resolve clearly signals from the protons on the methyl groups of methanol, O-methylisourea, and S-methylcysteine.

At a given pD value, the velocity of the reaction was pseudo first order with respect to the total concentration of O-methylisourea. Pseudo-first-order rate constants for decomposition of O-methylisourea in 1 M cysteine were obtained from the

slopes of linear -ln (O-methylisourea concentration) vs. time plots. When O-methylisourea was in excess, pseudo-first-order rate constants for S methylation of cysteine were obtained from the slopes of linear -ln (cysteine concentration) vs. time plots. The concentration of cysteine at a given time was obtained from the difference between the initial concentration of cysteine and the concentration of S-methylcysteine determined from the nuclear magnetic resonance signal.

The pH dependence of the rate of S methylation of 2-mercaptoethanol (0.1 M) by O-methylisourea (1.0 M) was studied at 28° in water. Reactions were run under a stream of nitrogen in 10⁻³ M EDTA solutions, which were previously degassed. A Radiometer TTT-lc pH-Stat was used to maintain constant pH. Pseudo-first-order rate constants for the disappearance of the thiol were obtained from linear plots of -ln (concentration of unreacted 2-mercaptoethanol) vs. time. The concentration of the thiol was determined by assaying aliquots from the reaction mixture using 5,5'-dithiobis(2-nitrobenzoic acid) according to the method of Ellman (1959). The rate of disappearance of 2-mercaptoethanol in the absence of O-methylisourea was less than 2% (except at pH 7 where it was 16%) of its rate of disappearance in the presence of O-methylisourea. For the run at pH 7, the pseudo-first-order rate constant for S methylation of 2-mercaptoethanol was estimated from the difference in rate constants for the disappearance of thiol in the presence and absence of O-methylisourea.

Identification of Products of the Reaction between O-Methylisourea and Cysteine. S-Methylcysteine and methanol were identified as the products of reaction from the correspondence of the nuclear magnetic resonance spectra of the products with the nuclear magnetic resonance spectra of methanol and S-methylcysteine. S-Methylcysteine was also identified as a product from recoveries of S-methylcysteine sulfone after oxidation of the product mixture with performic acid according to the method of Moore (1963). Amino acid analyses of the oxidized reaction mixture yielded cysteic acid, S-methylcysteine sulfone, and serine. The S-methylcysteine sulfone appeared in the same position on the chromatogram as S-methylcysteine sulfone produced by performic acid oxidation of S-methylcysteine. Some serine (10%) and cysteic acid (4%) were also produced on oxidation of S-methylcysteine with performic acid. Amino acid analyses were determined by the method of Spackman *et al.* (1958) with the exception that, the absorbance at 570 mμ was measured continuously on a Gilford Model 2000 multiple-sample absorbance recorder with 0.5- and 1-cm flow-through cells. Formation of α-guanidino groups was demonstrated using the Sakaguchi reaction as modified by Weber (1930). Because the color yield of the Sakaguchi reaction is sensitive to thiol groups (Weber, 1930), this reaction could not be used to determine the concentration of α-guanidinocysteine.

Results

Guanidination and S methylation of cysteine by O-methylisourea could be followed clearly from time dependencies of nuclear magnetic resonance spectra, since the signals from the methyl protons on the methyl groups of O-methylisourea, methanol, and S-methylcysteine are distinguishable.

TABLE II: Rate Constants for S Methylation of 2-Mercaptoethanol by *O*-Methylisourea.^a

pH	10 ³ <i>k_M</i> ^b (min ⁻¹ M ⁻¹)	
	Obsd	Calcd ^c
7.01	0.070	0.081
8.00	0.70	0.74
9.00	3.9	4.2
9.60	6.0	6.0
10.02	5.6	5.1
11.06	1.4	1.0

^a At 28°, 10⁻³ M EDTA. Initial concentration of 2-mercaptoethanol, 0.1 M; initial concentration of *O*-methylisourea 1.0 M. ^b *k_M* represents the apparent second-order rate constant for S methylation. ^c Calculated using the relationship

$$k_M = k_{ME}^0 \frac{\alpha_H}{\alpha_H + K_I \alpha_H + K_E}$$

with $k_{ME}^0 = 1.1 \times 10^{-2} \text{ min}^{-1} \text{ M}^{-1}$. The apparent dissociation constant of *O*-methylisourea in water ($K_I = 8.8 \times 10^{-11}$) was determined from pH values of partially neutralized solutions of 1 M *O*-methylisourea. The apparent dissociation constant of 2-mercaptoethanol ($K_E = 7.25 \times 10^{-10}$) was estimated from pH values of partially neutralized solutions of 0.1 M 2-mercaptoethanol in 1 M Na₂SO₄.

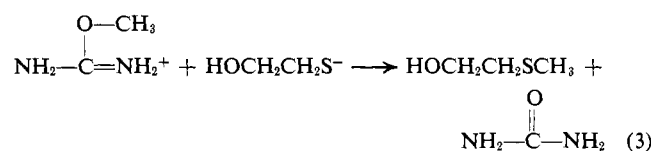
At pD 8.9 and 28°, chemical shifts (with respect to tetramethylsilane as an external standard) of δ 4.02, 3.37, and 2.17 were observed for *O*-methylisourea, methanol, and *S*-methylcysteine, respectively. Rates of decomposition of *O*-methylisourea (initial concentration 0.1 M) in solutions of 1 M cysteine are pseudo first order with respect to the total concentration of *O*-methylisourea at a given pD value. Studies of this reaction at different initial concentrations of cysteine and *O*-methylisourea indicated that the reaction is first order with respect to the concentrations of cysteine and *O*-methylisourea. Examination of Table I reveals the pD dependencies of the apparent second-order rate constants for formation of *S*-methylcysteine and methanol. Rate constants for formation of *S*-methylcysteine and methanol were obtained from the product of the pseudo-first-order rate constant for the decomposition of *O*-methylisourea (initial concentration 0.1 M) in solutions of 1 M cysteine and the fraction of *O*-methylisourea which decomposed to *S*-methylcysteine and methanol, respectively. Apparent second-order rate constants for guanidination were estimated from differences between the rate constants for formation of methanol in the presence and absence of cysteine.

The following observations suggest that guanidination of the α -amino group of cysteine is associated with the formation of methanol. (1) Reaction of 2-mercaptoethanol (1 M) with *O*-methylisourea (0.1 M) for 24 hr does not lead to the formation methanol, although S methylation of 2-mercaptoethanol occurs (eq 3). (2) After 24 hr at pD 10.9 a reaction mixture initially containing 1 M cysteine and 0.1 M *O*-methylisourea gives a positive Sakaguchi reaction.

TABLE III: Reactivity of Some Amino Acids toward *O*-Methylisourea.^a

Amino Acid	Ratio of Initial Concn of Amino Acid/ <i>O</i> -Methyl- isourea	Fraction of Minor Component Unreacted ^b
Arginine	10	0.91
Histidine	0.1	0.76
Lysine	10	0.36
Serine	10	0.56
Threonine	0.1	0.82
Aspartic acid	10	1.00
Asparagine	0.1	0.81
Glutamic acid	10	1.00
Glutamine	0.1	0.83
Methionine	0.1	0.78
Glycylglycine	10	0.19
Cysteine	10	Not detectable ^c

^a After 22 hr at pD 8.9, 28°, 10⁻³ M EDTA. Initial concentration of major component 1.0 M; initial concentration of minor component (0.1 M). ^b When *O*-methylisourea was the minor component, this fraction could be calculated from initial concentrations and the nuclear magnetic resonance signal from the methyl protons of *O*-methylisourea, or methanol. In some cases, only one signal could be detected because of interference with signals from the amino acid. When the amino acid was the minor component, this fraction was calculated from the nuclear magnetic resonance signal of the methyl protons in methanol. ^c At this pD value, S methylation of cysteine is ten times faster than guanidination.



(3) In 24 hr, excess *O*-methylisourea (2 M) S methylates over 86% of the thiol groups of cysteine (initial concentration 0.1 M) at pD 10.4. At pD 10.4, formation of methanol is faster than S methylation. This result suggests that formation of methanol does not involve loss of the free thiol group of cysteine.

The pH dependency of the second-order rate constant for S methylation of 2-mercaptoethanol is listed in Table II. As in the S methylation of cysteine, the second-order rate constant for S methylation of 2-mercaptoethanol also goes through maximum value.

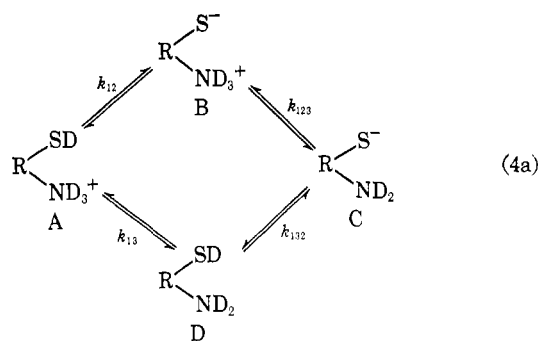
Reactivities of several amino acids toward *O*-methylisourea are compared in Table III. At pD 8.9, S methylation of the thiol group of cysteine is clearly faster than guanidination of the amino groups of the other amino acids and the unhindered peptide glycylglycine.

No reaction other than guanidination of an amino group could be detected from nuclear magnetic resonance spectra, when *O*-methylisourea was incubated for 24 hr at pD 8.9

with arginine, asparagine, aspartic acid, glutamine, glutamic acid, histidine, methionine, threonine, or serine. Furthermore, imidazole (1 M), ethanol (1 M), or acetate (1 M) failed to react (as judged from the constancy of nuclear magnetic resonance spectra) with *O*-methylisourea (initial concentration 0.1 M) after 24 hr at pD 8.9.

Discussion

The dependency of the observed second-order rate constant (k_M) for S methylation of cysteine on the deuterium ion activity (α_D) (Table I) can be rationalized by assuming that S methylation of cysteine occurs *via* nucleophilic displacement by a thiol anion on the methyl group of *O*-methylisouronium cation. As shown in eq 4, the ionization of



$$K_2 = k_{12} + k_{13} \quad (4b)$$

$$K_2 K_3 = k_{12} k_{123} = k_{13} k_{132} \quad (4c)$$

cysteine leads to the formation of two thiol anions (B and C). K_2 and K_3 represent the apparent dissociation constants for the removal of the second and third deuterium ions from cysteine. Since the extent of ionization of the thiol group in cysteine can be estimated spectrophotometrically, the microscopic equilibrium constants (k_{12} , k_{13} , k_{123} , and k_{132}) associated with the ionization of cysteine can be determined. For example, Benesch and Benesch (1955) followed the ionization of the thiol group in cysteine in water from changes in ultraviolet spectra and Elson and Edsall (1962) followed the ionization of the thiol group in cysteine from changes in Raman spectra. Both groups obtained a value of about two for the ratio k_{12}/k_{13} . The finding that k_{12} and k_{13} are within the same order of magnitude (*i.e.*, substantial concentrations of both thiol anions are formed) suggests that the reactivity of both thiol anions toward *O*-methylisouronium ion should be considered. Equation 5 represents the dependency of the observed second-order rate constant for S methylation on the deuterium ion activity.

$$V = k_M[\text{RSD}_i][\text{RI}_i] = (k_{MB}^0[B] + k_{MC}^0[C])[\text{RID}^+] \quad (5a)$$

$[\text{RSD}_i]$ and $[\text{RI}_i]$ represent the total concentration of cysteine thiol groups and *O*-methylisourea, respectively.

$$k_M = \frac{\left(k_{MB}^0 + \frac{k_{MC}^0 k_{123}}{[\alpha_D]} \right) k_{12}}{\left(1 + \frac{K_2}{[\alpha_D]} + \frac{K_2 K_3}{[\alpha_D]^2} \right) ([\alpha_D] + K_1)} \quad (5b)$$

The values of K_2 (3.2×10^{-9}) and K_3 (1.8×10^{-11}) were determined from pD values of partially neutralized 1 M cysteine, whereas k_{12} (2.4×10^{-9}) was estimated from the fraction of thiol in the anionic form (α_s) at several apparent deuterium ion activities (α_D), as estimated using the relationship $\text{pD} = -\log \alpha_D$ in 1 M glycine and the relationship (eq 6) given by Edsall and Wyman (1958).

$$k_{12} = \alpha_s([\alpha_D] + K_2) - (1 - \alpha_s) \frac{K_2 K_3}{[\alpha_D]} \quad (6)$$

The fraction of thiol anion was determined spectrophotometrically using the method of Benesch and Benesch (1955). Values of 0.7×10^{-9} , 2.4×10^{-11} , and 8.2×10^{-11} for k_{13} , k_{123} , and k_{132} , respectively, were obtained from the relationships in eq 4a and 4b. The apparent dissociation constant of *O*-methylisouronium ion ($K_1 = 4.0 \times 10^{-11}$) was determined from pD values of partially neutralized 0.1 M *O*-methylisourea. As shown in Table I, eq 5b describes the pD dependency of k_M reasonably well.

The dependency of the observed second-order rate constant (k_M) for S methylation of 2-mercaptoethanol on the hydrogen ion activity (α_H) can also be rationalized by assuming that S methylation of 2-mercaptoethanol occurs *via* nucleophilic displacement by thiol anion on the methyl group of *O*-methylisouronium cation. Thus, eq 7 describes the dependency of k_M on the hydrogen ion activity (Table II).

$$V = k_M[\text{RSH}_i][\text{RI}_i] = k_{ME}^0[\text{E}][\text{RIH}^+] \quad (7a)$$

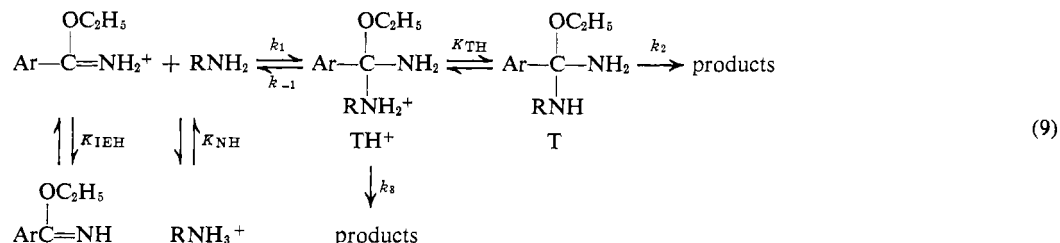
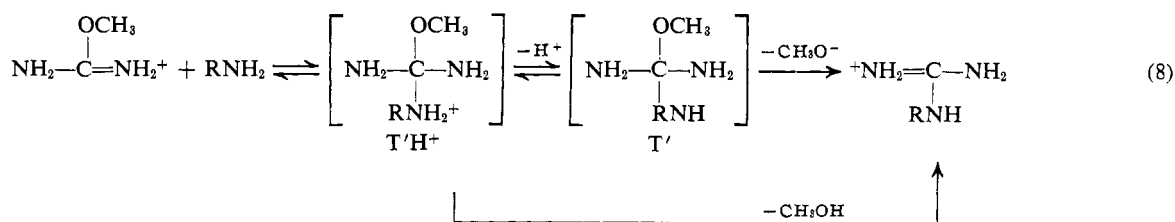
$$k_M = k_{ME}^0 \frac{\alpha_H}{\alpha_H + K_1} \frac{K_E}{\alpha_H + K_E} \quad (7b)$$

$[\text{RSH}_i]$ and $[\text{E}]$ represent the total concentration of 2-mercaptoethanol and the concentration of thiolate anion, respectively. K_E represents the acid dissociation constant of 2-mercaptoethanol. As expected, the apparent second-order rate constants for the reaction between *O*-methylisouronium cation and the thiolate ions B and C and the conjugate base of 2-mercaptoethanol (E) are similar in magnitude. (See the values of k_{MB}^0 , k_{MC}^0 , and k_{ME}^0 in Tables I and II.)

Guanidination of amino groups by *O*-methylisourea probably proceeds *via* a two-step addition elimination reaction (eq 8). Convincing evidence for such a two-step reaction was presented by Hunter and Ludwig (1962) and by Hand and Jencks (1962) in their classical studies of reactions of imido esters with amines. Hand and Jencks (1962) showed that amidination of strongly basic amines probably proceeds mainly through the pathway depicted in eq 9.

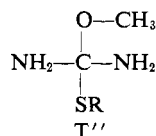
Above pH 7, the products form mainly through decomposition of T rather than TH^+ . At pH values between 7 and 9, elimination of ethanol from tetrahedral intermediate T is rate controlling, whereas at higher pH values formation of the intermediate is rate controlling.

This change in the rate-determining step was reflected by the dependency of the apparent second-order rate constant for amidination on the hydrogen ion concentration, because the transition states for the rate-controlling steps had different charges. The dependency of the apparent second-order rate constant for amidination on the hydrogen ion activity for the scheme depicted in eq 9 is given by eq 10 (Hand and Jencks, 1962). Equation 10 should also describe the depen-



dency on the deuterium ion activity of the apparent second-order rate constant for guanidination of an amino group by *O*-methylisourea. Because of changes in reactivity (of reactants and intermediates) accompanying ionization of the thiol group in cysteine, the relationship describing the apparent second-order rate constant's dependency on the deuterium ion activity contains too many adjustable parameters for it to be useful in interpreting the pD-rate profile for guanidination of cysteine.

It is not unexpected that thiol anions and amines react differently with *O*-methylisouronium ion. Formation of an isothiuronium ion from an *O*-methylisouronium ion and a thiol anion seems unlikely, since such a reaction would undoubtedly involve the elimination of methoxide anion rather than thiol anion from a tetrahedral intermediate such as T'. The difficulty in expelling a basic leaving group from a tetrahedral intermediate containing another less basic group as an alternate leaving group has been discussed by Bruice and Schmir (1957). Also Kirsch and Jencks (1964) have shown that the ability of imidazole to displace groups from carbonyl carbon decreases sharply as the pK_a of the leaving group becomes larger than 10.



Although the reactivity of unhindered nucleophiles with carbonyl carbon correlates roughly with the basicity of the nucleophile (when the nucleophile is more basic than the leaving group), nucleophilic reactivity of bases toward saturated carbon in S_N2 reactions is correlated with polarizability and other measures of the nucleophile's ability to interact with saturated carbon at a distance. (For an excellent discussion of factors influencing nucleophilic reactivity, see Jencks (1969).) Sulfide anions are more polarizable than amines, and have been found to be more reactive as nucleophiles in displacements on saturated carbon. There is, however, a large dependency of nucleophilicity on medium effects (especially for charged nucleophiles), as well as the nature of the substrate, and steric effects. For example,

$$k = \frac{k_1 \left[1 + \frac{k_2 K_{\text{TH}}}{k_3 [\alpha_{\text{H}}]} \right]}{1 + \frac{k_{-1}}{k_3} + \frac{k_2 K_{\text{TH}}}{k_3 [\alpha_{\text{H}}]}} \frac{K_{\text{NH}}}{[\alpha_{\text{H}}] + K_{\text{NH}}} \frac{[\alpha_{\text{H}}]}{[\alpha_{\text{H}}] + K_{\text{IEH}}} \quad (10)$$

phenyl sulfide anion (pK_a = 6.5) is 20,000 to 30,000 times more reactive than aniline (pK_a = 4.6) or ammonia (pK_a = 9.2) toward methyl iodide in methanol at 25° (Pearson *et al.*, 1968). On the other hand, hydrogen sulfide anion (pK_a = 6.8) is only 32 times more reactive than pyridine (pK_a = 5.2) and four times more reactive than aniline toward methyl bromide in water at 25° (Swain and Scott, 1953). Thus, it is difficult to predict just how much more reactive than an amino group, a thiol anion should be in an S_N2 displacement on the *O*-methylisouronium ion in D₂O.

The high reactivity at pD 8.9 of *O*-methylisourea toward a thiol group relative to its reactivity toward other functional groups present in proteins suggests that *O*-methylisourea should prove to be a useful reagent for selectively alkylating thiol groups in proteins. The occurrence of S methylation at appreciable rates at pD 10–12 suggests that conditions normally used to guanidinate a protein (*i.e.*, incubation of a protein for long periods of time with concentrated solutions of *O*-methylisourea) could lead to substantial S methylation of thiol groups present in the protein.

Acknowledgments

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Nonpolar Effects in Reactions of the Sulfhydryl Group of Papain*

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ABSTRACT: A series of *N*-alkylmaleimides, *N*-ethylmaleimide, and *N*-butylmaleimide to *N*-decylmaleimide, inclusive, were shown to inactivate papain effectively at neutral pH through a pseudo-first-order process.

The apparent second-order rate constants for papain inactivation were demonstrated to increase with increasing chain length of the maleimide derivatives. Titration of the enzyme with *N*-pentylmaleimide indicated that only one functional group of the enzyme reacted with the maleimide and a 1:1 stoichiometry existed between the modification of this functional group and the loss of catalytic activity. Saturation kinetics were obtained in the maleimide inactivation of papain and the absence of chain-

length effects in the maximum rate of inactivation with four different *N*-alkylmaleimides indicated that the involvement of alkyl side chains in the overall inactivation process occurs predominantly in the binding of maleimides to the enzyme. The maleimide inactivation of papain was described as a two-step process, the binding of maleimides at the active site which is facilitated by nonpolar interactions of the alkyl side chains of the maleimide derivatives with the nonpolar cleft region of the enzyme followed by an irreversible inactivating reaction between the bound maleimide and the active-site sulfhydryl group. α -*N*-Benzoyl-L-arginine, a substrate-competitive inhibitor of papain, was shown to provide some protection against maleimide inactivation.

The papain-catalyzed hydrolysis of a number of substrates has been reported to proceed through the formation of an acyl-enzyme intermediate. Smith *et al.* (1955) indicated that the acyl-papain intermediate is a thiol ester involving the essential sulfhydryl group of the enzyme. Several more recent studies have contributed evidence in confirmation of this process (Bender and Brubacher, 1964; Lowe and Williams, 1965; Kirsch and Igelstrom 1966). Inactivation of papain by alkylation was attributed to specific modification of the essential cysteine residue of the enzyme (Balls and Lineweaver, 1939; Finkle and Smith, 1958; Hollaway *et al.*, 1964; Light, 1964; Sluyterman 1968). In peptide-sequencing studies, Light *et al.* (1964) demonstrated the active-site sulfhydryl and only sulfhydryl of the enzyme to be Cys-25. X-Ray studies of crystalline papain (Drenth *et al.*, 1968) have revealed that the active-site cysteine residue lies in a nonpolar cleft region of the enzyme. Recent studies of the papain sulfhydryl group (Sluy-

terman, 1968; Chaiken and Smith, 1969a; Chaiken and Smith, 1969b) have demonstrated that other ionizable groups of the enzyme can influence reactions of the essential sulfhydryl group. The present study provides evidence that the nonpolar cleft region of the enzyme can also affect reactions of the active-site sulfhydryl group.

Experimental Section

Materials. Twice-crystallized papain suspended in 0.05 M sodium acetate, pH 4.5 (19 Bz-L-AgEt units per mg), *N*-carbobenzoylglycine *p*-nitrophenyl ester, and α -*N*-benzoyl-L-arginine were obtained from the Sigma Chemical Co. Stock solutions of the enzyme were prepared by diluting the crystalline enzyme suspension with 0.02 M potassium phosphate, pH 6.8. Stock solutions of the substrate, *N*-carbobenzoylglycine *p*-nitrophenyl ester (1.5×10^{-2} M), were prepared in acetonitrile. *N*-Ethylmaleimide was purchased from Eastman Organic Chemicals. *N*-Butylmaleimide was obtained from Nutritional Biochemical Corp. The *N*-pentyl-, *N*-hexyl-, *N*-heptyl-, *N*-octyl-, *N*-nonyl-, *N*-decyl-, and *N*-phenylmaleimides and the *N*-hexyl-, *N*-octyl-, and *N*-phenylmaleamic acids were prepared according to Heitz *et al.* (1968).

Methods. Activation of papain (1.86×10^{-6} M) was carried

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