Inactivation of Papain by S-Methylation of Its Cysteinyl Residue with *O*-Methylisourea*

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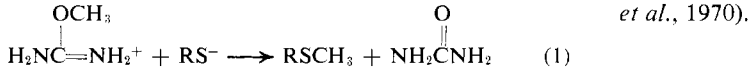
ABSTRACT: *O*-Methylisourea (OMI), a reagent routinely used to guanidinate amino groups in proteins, inactivates papain by S-methylating the thiol group of Cys-25, the only thiol group in papain. S-Methylation of Cys parallels inactivation. The rate of guanidination of lysyl residues at pH 7 is too slow to account for inactivation. Inactivation of papain by OMI is first order with respect to the concentration of active papain remaining. The dependence of the apparent second-order rate constant for inactivation on the hydrogen ion activity is that which is expected for attack of a thiol anion on *O*-methylisouronium ion. A value of 8.15 for the pK_a of the thiol group at the active site of papain can be estimated from the pH dependence of the rate of inactivation. The intrinsic rate constant (0.8×10^{-2} min^{-1} M^{-1}) is strikingly similar to the value of 1.1×10^{-2} min^{-1} M^{-1} observed for the second-order rate constant for the attack of the thiol anion of 2-mercaptoethanol on *O*-methylisouronium ion. This result suggests that functional groups in the vicinity of the active site of papain neither inhibit nor enhance the reaction between the thiol group of Cys-25 and OMI. Mercuripapain in which the thiol group of Cys-25 is presumably bound to mercury is not inactivated by OMI.

Our previous study of the facile methylation of organic thiols by *O*-methylisourea (OMI)[1] (Banks and Shafer, 1970) suggested that OMI, and possibly other isoureas, could be useful selective reagents for alkylating thiol groups in proteins (eq 1). Because of the reduced probability of altering

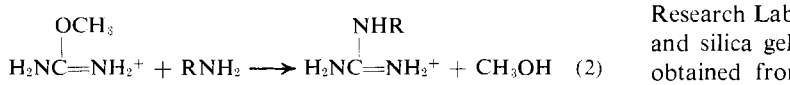


$$H_2NC(OCH_3)=NH_2^+ + RS^- \longrightarrow RSCH_3 + NH_2CONH_2 \quad (1)$$

protein structure or sterically blocking an active site upon alkylating a thiol group with the relatively small and uncharged methyl group, *O*-methylisoureas promise to be useful tools for revealing the role of thiols in the activity of proteins.

Methyl *p*-nitrobenzenesulfonate, a reagent which selectively S-methylates the thiol group in reduced lysozyme (Heinrikson, 1970), is another potentially useful S-methylating reagent for thiol groups in proteins.

Although OMI guanidinates primary amino groups (eq 2) at high pH values (*e.g.*, pH 10.5) more efficiently than it S-



$$H_2NC(OCH_3)=NH_2^+ + RNH_2 \longrightarrow H_2NC(NHR)=NH_2^+ + CH_3OH \quad (2)$$

methylates thiols, the reverse is true below pH 10 (Banks and Shafer, 1970). Surprisingly, however, despite extensive studies of the reaction of OMI with proteins over the past 22 years (Kimmel, 1967), this work is the first report of the methylation of a thiol group in a protein by OMI. In this work, the reaction of OMI with papain is characterized, and the feasibility of using OMI as a selective methylating reagent for thiol groups in proteins is demonstrated. Papain was chosen for this study, because it possesses a single thiol group, Cys-25, alkylation of which leads to loss of papain's catalytic activity (Light *et al.*, 1964; Husain and Lowe, 1969; Mitchel *et al.*, 1970).

Methods

Papain (EC 3.4.4.10), twice crystallized in suspension in 0.05 M acetate buffer (pH 4.5) (lots OEA, OJB, and 9JA), and mercuripapain (crystalline papain recrystallized in the presence of mercury) in suspension in 70% ethanol (lot OEA) were obtained from Worthington Biochemical Corp., Freehold, N. J. L-Cysteine was A grade from Calbiochem, Los Angeles, Calif. *O*-Methylisourea hydrogen sulfate was obtained from Aldrich Chemical Co., Milwaukee, Wis. *S*-Methylcysteine (SMC) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. *N*-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE), Mann Analyzed, was purchased from Mann Research Laboratories, New York, N. Y. 2-Mercaptoethanol and silica gel thin-layer chromatography plates (6061) were obtained from Eastman Kodak Co., Rochester, N. Y. 2-Aminoethanethiol hydrochloride and EDTA were purchased from Matheson Coleman & Bell, Norwood, Ohio. The distilled water supplied to the laboratory was passed through a demineralizer and redistilled in an all-glass still. Carbon dioxide and oxygen were removed from the water with a stream of nitrogen prior to all kinetic runs.

Measurements of pH were made using a Radiometer Model 4b pH meter or a Radiometer TTT-1c 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.

Amino acid analyses were determined by the method of Spackman *et al.* (1958) except that after development of the color, the absorbance at 570 nm was measured continuously

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[1] Abbreviations used are: OMI, *O*-methylisourea; BAEE, *N*-benzoyl-L-arginine ethyl ester; BA, *N*-benzoyl-L-arginine; SMC, *S*-methylcysteine.

on a Gilford Model 2000 multiple sample absorbance recorder with 0.5- and 1-cm flow-through cells. Areas under peaks were measured with a compensating polar planimeter sold by Gelman Instruments Co., Ann Arbor, Mich. The amino acid analyzer was calibrated with an 18 amino acid standard solution from Calbiochem.

Activation of papain (6.7×10^{-5} M) was carried out in the presence of 1.5×10^{-3} M activator and 3.0×10^{-4} M EDTA at pH 3.5, 25°. The activators used were Cys, 2-mercaptoethanol, and 2-aminoethanethiol. Cys and 2-aminoethanethiol were equally effective in the activation of papain as shown by Sanner and Pihl (1963), and in these experiments were much better than mercaptoethanol for conservation of enzyme activity over prolonged periods. The enzyme was activated at least 35 min, but not over 60 min prior to use in all experiments.

Activation of mercuripapain was achieved with 10^{-2} M 2-aminoethanethiol and 10^{-3} M EDTA prior to assay. The assay procedure used was that given below for papain. Mercuripapain was treated at pH 10.5 with OMI in a modification of the procedure described by Shields *et al.* (1959) for guanidination, and reacted with OMI at pH 8.5 under conditions described below for papin.

Routine assays of the catalytic activity of papain were performed by following the papain-catalyzed hydrolysis of BAEE spectrophotometrically using a Gilford Model 240 recording spectrophotometer. Sample and reference chambers were maintained at 25 ± 0.1° with water from a constant-temperature circulator. For routine assays, 3.0 ml of pH 5.15 acetate buffer (0.0365 M sodium acetate–0.0135 M acetic acid) was allowed to equilibrate in a cuvet in the thermoregulated cell compartment; to this was added 0.100 ml of enzyme solution with mixing followed by 0.100 ml of BAEE with mixing, after which time, the linear time dependence of absorbance at 255 nm was recorded for at least 5 min. For assays in which the enzyme solution had a pH >9.5, 0.1 ml of concentrated acetate buffer (1.095 M sodium acetate–0.405 M acetic acid) was added to the cuvet prior to adding the enzyme solution. Typical concentrations in the cuvet during the assay were 8.9×10^{-7} M papain, 2.2×10^{-4} M Cys, 6.0×10^{-5} M EDTA, and 4.8×10^{-4} M BAEE. Stock solutions of BAEE (1.55×10^{-2} M) were prepared fresh daily. The ΔE_{255} (BAEE–BA) was determined to be 1204 M^{-1} cm^{-1} in acetate buffer at pH 5.15, Γ/2, 0.037 M, 25°. Similar values, ΔE_{253} 1071 M^{-1} cm^{-1}, in acetate buffer at pH 5.2, μ 0.3, 25° (Whitaker and Bender, 1965), and ΔE_{254} 1150 M^{-1} cm^{-1}, in phosphate buffer at pH 8, 25° (Rick, 1963), have been reported. Concentrations of papain (total protein) were determined from the absorbance at 280 nm using $E_{1\%}^{1\,cm} = 24.7$ (Bender *et al.*, 1966; Glazer and Smith, 1961) and a molecular weight of 23,350 (Wolthers *et al.*, 1970).

Treatment of Papain with OMI. Fresh stock solutions of activated papain were prepared for each experiment. The concentration of papain in the stock solution varied from 7.5×10^{-5} to 9.4×10^{-5} M. The desired quantity of OMI was dissolved in concentrated sodium hydroxide so that the pH would be approximately correct for the run. This solution was then added to sufficient solid EDTA and activator to obtain concentrations of 2×10^{-3} and 6.9×10^{-3} M, respectively, in the reaction mixture. The solution was adjusted to the desired pH using 2 N sodium hydroxide, an aliquot of the enzyme stock solution was added, carbon dioxide and oxygen were removed with a stream of nitrogen, and the solution was covered with parafilm. The temperature during all runs was maintained at 26 ± 0.5° by using circulating water from a Landa U3 water bath. The buffering capacity of the reaction mixture was sufficient to maintain the pH within 0.05 pH unit except in the run at pH 7. The pH was maintained at pH 7 with a Radiometer TTT-1c pH-Stat during this run. Unless stated otherwise, concentrations of OMI and papain in the reaction mixture were 1 M and 2.5×10^{-5} to 3.1×10^{-5} M, respectively.

Kinetics of Inactivation. At a given pH value the velocity of the inactivation reaction was pseudo first order with respect to the total concentration of active enzyme remaining. The pseudo-first-order rate constants for inactivation were obtained from the difference in rates of inactivation in the presence and absence of OMI. The rate of inactivation of papain in the absence of OMI was never more than 6% of the rate of inactivation with OMI present. Runs performed to determine the rate of inactivation of enzyme in the absence of OMI were made 1 M in sodium sulfate. For each time point, two 0.1-ml aliquots were removed from the reaction mixture. One of these aliquots was assayed immediately as described above for routine assays. The other aliquot was added to 3.0 ml of aqueous solution containing 1×10^{-2} M activator and 3.3×10^{-3} M EDTA. To this solution was added concentrated acetate buffer, 0.1 ml for runs of pH ≤9.5 and 0.2 ml for runs of pH >9.5. Carbon dioxide and oxygen were removed from the solution with a stream of nitrogen. The solution was then covered with parafilm and incubated for 30 min at 25°, after which time it was transferred to a cuvet, 0.1 ml of BAEE was added, and the rate of change of absorbance at 255 nm was recorded as above. The pseudo-first-order rate constants for inactivation of papain obtained from the slopes of the linear −ln (papain activity remaining) *vs.* time plots for the two assays agreed within 5% or less in all cases, in both presence and absence of OMI.

The velocity of the methylation reaction was determined at pH 8.5 and was also found to be pseudo first order. Samples were analyzed for SMC and assayed for activity. In these runs, 2-aminoethanethiol was used as activator. Aliquots for SMC analyses were adjusted to pH 2.7–2.8 with concentrated HCl and stored at 5° for 30 min. The precipitated protein was centrifuged and washed three times with 10% NaCl. The supernatant from the final washing gave a negative barium test for sulfate indicating that removal of soluble material was virtually complete. Removal of small molecules from the precipitated protein is essential for an accurate determination of SMC. Residual OMI promotes destruction of SMC during hydrolysis in 6 N HCl; however, no loss was observed under normal reaction conditions during incubation of a mixture of OMI and SMC. Cys released during activation of papain (Glazer and Smith, 1965; Sluyterman, 1967; Klein and Kirsh, 1969a) forms SMC which must be removed or high values of SMC will be obtained on analysis.

The centrifuged protein was hydrolyzed in 6 N HCl at 105° for 18 hr in an evacuated sealed tube. The hydrolysate was taken to dryness on a rotary evaporator and the residue was taken up in 2 drops of distilled water. Prior to amino acid analysis, the hydrolysates were subjected to thin-layer chromatography on silica gel in order to ensure separation of SMC from Glu, Pro, and Cys. Amino acid analysis of prepared solutions of Glu, Pro, Cys, and SMC indicated that Cys and SMC elute in a position which overlaps Pro and that the presence of a large amount of Glu distorts the SMC peak. Moreover, when the amount of SMC in comparison to the amount of Glu and Pro is small, as in the case in papain, the quantitation becomes difficult. Papain contains 20 Glu + Gln residues, 10 Pro residues (Hussain and Lowe, 1969), and 0.4 residue of Cys. The Cys content of commercially prepared

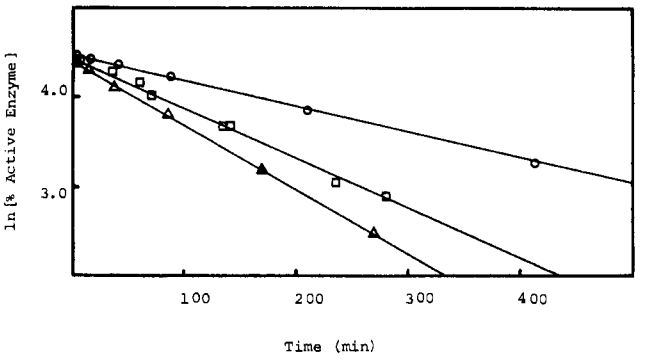

FIGURE 1: Pseudo-first-order plots for inactivation of papain by 1 M OMI at 26°. (○) pH 8.0, (□) pH 8.5, and (△) pH 9.5.

papain is ~40% (Glazer and Smith, 1965; Klein and Kirsh, 1969a,b; Finkle and Smith, 1958).

Prior to amino acid analysis, the aqueous solution from acid-hydrolyzed methylated papain was spotted in a line 14 cm long, 2 cm from the bottom, and centered on two 20 × 20 cm silica gel thin-layer chromatography plates. These sheets were placed back to back and eluted simultaneously in an Eastman chromatogram chamber plate set with chamber plates held perpendicular to the solvent reservoir. The chromatograms were eluted for 60 min in 1-propanol–34% ammonium hydroxide (7:3, v/v). The bands were located by removing strips from the sides of the dry chromatograms and spraying with a cadmium–ninhydrin reagent (0.5 g of ninhydrin in 100 ml of acetone plus 0.25 g of $CdCl_2$ in 2.5 ml of glacial acetic acid and 5 ml of water) followed by heating at 100°. The band containing SMC was cut from the sheet and eluted with 3 ml of water. The eluted strip did not produce a color reaction with cadmiun–ninhydrin reagent indicating complete elution of amino acids. The eluent was taken to dryness on a rotary evaporator and the residue dissolved in pH 2.2 citrate buffer (Moore and Stein, 1954). Amino acid analysis yielded SMC, Val, Ile, Leu, Tyr, Phe, and a trace of a substance which cochromatographs with urea.

Since Phe was quantitatively removed from the thin-layer chromatogram along with SMC, Phe was used as the basis for quantitation of SMC. The ninhydrin color yield of SMC was 69% of Ala as determined from pure samples of Ala and SMC.

Occasionally, some of the SMC (<50%) was oxidized to *S*-methylcysteine sulfoxides and/or *S*-methylcysteine sulfone during thin-layer chromatography. These products of oxidation of SMC were eluted with SMC from the thin-layer chromatogram, and appeared as a single peak on the amino acid analyzer in a position between cysteic acid and Asp. The yield of *S*-methylcysteine was corrected for oxidation during thin-layer chromatography by adding the yield of oxidation products to the amount of *S*-methylcysteine.

Recovery of papain from the precipitation step was 85–93% as judged by amino acid analysis and absorbance readings at 280 nm. Overall recovery of papain after thin-layer chromatography was 50–70%, as judged from the recovery of Phe.

Results

Figure 1 reveals that OMI inactivates papain, and that inactivation is a pseudo-first-order process with respect to the concentration of active enzyme, when the concentration of OMI is high relative to the concentration of papain. Observed

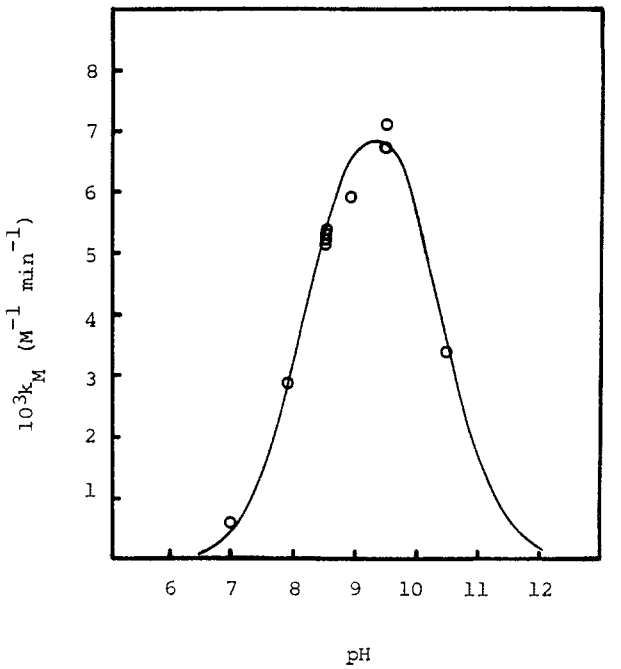

FIGURE 2: pH dependence of the observed rate constant for inactivation of papain in solutions of 1 M OMI. Solid line calculated using eq 3 with K_I, K_E, and k_M^0 set at 3.98×10^{-11}, 7.08×10^{-9}, and 0.8×10^{-2} min^{-1} M^{-1}, respectively.

rates of inactivation of papain are unaltered by incubating diluted reaction mixtures of OMI and papain with added thiols and EDTA prior to assaying for enzymic activity. This result shows inactivation is not caused by reversible oxidation of the active site thiol group (to a disulfide, for example). Separate studies of the dependence of the observed pseudo-first-order rate constant on the concentration of OMI indicated that the rate of inactivation is only roughly first order with respect to the concentration of OMI. For example, when the OMI concentration is decreased from 1.0 to 0.5 M, the rate of inactivation decreases by a factor of 1.6. Because of decomposition of OMI at concentrations greater than 1 M (*via* polymerization of OMI) and because of the possibility of the existence of specific salt effects on the rate of inactivation of papain at high concentrations of OMI, it is difficult to determine whether the deviation of the rate of inactivation from a first-order dependence on the concentration of OMI reflects formation of a papain–OMI complex prior to inactivation of papain.

Thus, the inactivation of papain was studied at a constant concentration of OMI (1 M); however, the rate constants are reported as if inactivation were a second-order process. The pH dependence of the apparent second-order rate constants for inactivation is given in Figure 2. The bell-shaped pH–rate profile is characteristic of reactions between OMI and ionizable nucleophiles, and is consistent with the idea that reaction occurs when the basic form of the nucleophile attacks the *O*-methylisouronium cation.

During modification of papain with OMI, no loss of any aminoacyl residue but Lys and Cys could be detected. Also, analyses for methylhistidines were negative. The close correspondence between rates of conversion of cysteine to *S*-methylcysteine and the loss of enzymic activity (Table I) suggest that inactivation is caused by S-methylation of Cys-25 (the only cysteinyl residue in papain).

Although the rate of modification of a lysyl residue is much

smaller than the rate of methylation of a cysteinyl residue at pH 8.5 (Banks and Shafer, 1970), the 25-fold excess of lysyl residues over cysteinyl residues in the papain sample used could be responsible for our observing the guanidination of lysyl residues during the inactivation reaction at pH 8.5 (Table I). Since more than one lysyl residue is guanidinated, the possibility that inactivation is being caused by the modification of a reactive lysyl residue must be considered. In an attempt to resolve this point, reaction of OMI with mercuripapain was studied.

Table I shows that mercuripapain is not inactivated by OMI, whereas activated mercuripapain (in which added thiol and EDTA remove the mercuric ion from the thiol group in papain) is readily inactivated by OMI. Table I also illustrates an apparent increase in reactivity of the lysyl residues accompanying removal of the mercuric ion. This result is consistent with the idea that mercuric ion blocks attack of OMI on a lysyl residue near the active site of papain. The possibility that guanidination of this lysyl group is responsible for the inactivation of papain can be excluded, since the guanidination of lysyl residues at pH 7 is too slow to account for inactivation of papain (Table I).

TABLE I: Reaction of Papain with 1 M OMI at 26°.

Sample	Fractional Loss in Catalytic Efficiency	Moles of Homoarginine[a]/Mole of Papain	Moles of SMC/Mole of Papain
Papain at pH 8.5	0.08		0.0
	0.30		0.3
	0.66		0.7
	1.00[b]		1.0
	1.00[c]	6.0	1.0
Mercuripapain at pH 8.5	0.00[d]	1.9	0.0
Activated mercuripapain at pH 8.5	1.00[d]	2.9	1.0
Activated mercuripapain at pH 7.0	0.6	0.1	0.5

[a] The concentration of papain was determined from the cysteine content, that is, the maximum yield of SMC. The cysteine content of the papain used indicated it was 40–45% pure. This result is an agreement with results of other workers (*e.g.*, Glazer and Smith, 1965; Klein and Kirsh, 1969a,b; Finkle and Smith, 1958). The error in the determination of moles of homoarginine and SMC is estimated to be $<\pm 0.1$ residue. [b] After 15 hr. [c] After 13 hr at 40°. [d] After 13.5 hr.

Discussion

The close correlation between formation of *S*-methylcysteine and inactivation of papain (Table I), coupled with guanidination of lysyl residues being too slow at pH 7 to account for inactivation and the absence of any other observable reaction between papain and OMI, strongly suggest that OMI inactivates papain by S-methylating Cys-25. Since the relatively small methyl group is unlikely to sterically block the active site of papain, it is reasonable to conclude that the thiol group of Cys-25 must lose its proton during the catalytic cycle. This requirement is of course consistent with the observation of the buildup and decay of acyl-enzyme intermediates during papain catalyzed reactions (*e.g.*, Brubacher and Bender, 1966).

Assuming the thiol anion of Cys-25 in papain reacts with the conjugate acid of OMI to form an inactive derivative of papain, eq 3 should describe the dependency of the observed second-order rate constant (k_M) on the hydrogen ion activity (α_H) provided that: (a) any interaction between OMI and

$$k_M = k_M^0\left(\frac{\alpha_H}{\alpha_H + K_I}\right)\left(\frac{K_E}{\alpha_H + K_E}\right) \quad (3)$$

papain which may precede S-methylation (*e.g.*, formation of a papain–OMI complex) is not pH dependent and (b) ionization of other groups on the enzyme does not alter the bascity or reactivity of the thiol group.

Values of 10.4, 8.15, and 0.8×10^{-2} min^{-1} M^{-1} for pK_I, pK_E, and k_M^0 gave the best fit of eq 3 to the experimental data. The p*K* of *O*-methylisouronium ion (pK_I) is in tolerable agreement with the value of 10.05 determined previously by titration of OMI (Banks and Shafer, 1970). The value of pK_E is consistent with other studies of the inactivation of papain with neutral alkylating agents. For example, the inactivation of papain by L-1-chloro-3-tosylamido-4-phenyl-2-butanone (Bender and Brubacher, 1966) and D- and L-α-iodopropionamide (Wallenfels and Eisele, 1968) is dependent on a group on the enzyme with a p*K* of 8.0–8.3 being in the basic form. The intrinsic second-order rate constant (k_M^0) for inactivation of papain is almost identical with the value of 1.1×10^{-2} min^{-1} M^{-1} for the attack of the thiolate anion of 2-mercaptoethanol on protonated OMI (Banks and Shafer, 1970). Although the thiol anion of 2-mercaptoethanol (pK = 9.14) is about tenfold more basic than the thiol anion on the enzyme, this difference in basicity would not be expected to lead to a difference in reactivity toward OMI, since the nucleophilicity of a given group is a very insensitive function of its basicity. (See Jencks, 1969, for a discussion of factors affecting nucleophilicity). Also, the rate constants for attack of the thiol anion of cysteine (1.3×10^{-2} and 2.4×10^{-2} min^{-1} M in D_2O depending on whether the α-amino group is protonated or unprotonated) on protonated OMI are similar to that observed for 2-mercaptoethanol (Banks and Shafer, 1970). The close correspondence between the rate constants for the reaction of the thiol anion of Cys-25 in papain and the thiol anion of mercaptoethanol strongly suggests that the groups near the active site of papain neither prevent nor enhance the reaction between Cys-25 and OMI. This result is surprising, since the enhanced reactivity of α-halo acids with papain over their amide derivatives has been explained by assuming a positively charged group near Cys-25 aligns the negatively charged α-halocarboxylate favorably with respect to Cys-25 (Wallenfels and Eisele, 1968). Presumably this positive charge near Cys-25 should lead to reduced reactivity of Cys-25 toward cationic alkylating reagents such as OMI. Further studies are under way to resolve this apparent difference in the reactivity of Cys-25 toward anionic and cationic alkylating agents.

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Physicochemical Studies of the Relation between Structure and Function in Hemoglobin Hiroshima (HC3β, Histidine → Aspartate)*

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ABSTRACT: Various physicochemical properties of Hb Hiroshima (HC3β, histidine → aspartate) were studied to clarify the mechanism for the altered oxygen equilibrium functions of the hemoglobin and the role of the C-terminal histidine residues of the β chains. The cooperativity in oxygen binding of Hb Hiroshima is markedly diminished by stripping it of 2,3-diphosphoglycerate (DPG). The effect of DPG on the oxygen equilibrium of Hb Hiroshima is, however, apparently normal because its overall oxygen affinity exhibits the same dependence on the concentration of DPG as that of Hb A and the overall free energy of interaction among the binding sites of oxygen of Hb Hiroshima increases on the addition of DPG by an extent similar to that of Hb A. Ultraviolet difference spectra suggest that the conformational changes of the $\alpha_1\beta_2$ contacts that occur during oxygenation are impaired in Hb Hiroshima corresponding to its diminished cooperativity and that the penultimate tyrosine residues of the β chains in Hb Hiroshima undergo environmental changes similar to those which occur in Hb A during oxygenation. The intrinsic microscopic equilibrium constant for the fourth stage of oxygenation, k_4, for Hb Hiroshima is insensitive to the concentration of DPG and nearly equal to that for Hb A, whereas the constant for the first stage of oxygenation, k_1, decreases on the addition of DPG and is always larger than that of Hb A, irrespective of DPG concentration, indicating that deoxy-Hb Hiroshima probably assumes a more unconstrained form than deoxy-Hb A. The reaction rates of 4,4′-dipyridine disulfide with the sulfhydryl groups of oxy- and deoxy-Hb Hiroshima were more rapid than for Hb A, particularly that of the deoxy form. Electron paramagnetic resonance spectra of NO-Hb Hiroshima are identical with those of NO-Hb A.

Hemoglobin Hiroshima is a mutant hemoglobin with greatly altered oxygen equilibrium functions: it has an oxygen affinity 5-fold higher than that of Hb A[1] at pH 6.5, a decreased Bohr effect about half that of Hb A, and reduced heme–heme interaction (Hill's constant, 2.0–2.6, compared to 3.0 for Hb A) (Imai, 1968). The carriers of this hemoglobin have no apparent clinical symptoms save a mild erythrocytosis in com-

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