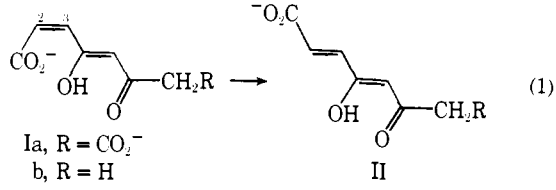
Maleylacetone *cis-trans*-Isomerase. Mechanism of the Interaction of Coenzyme Glutathione and Substrate Maleylacetone in the Presence and Absence of Enzyme[1]

Stanley Seltzer* and Mow Lin

Contribution from the Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973. *Received November 27, 1978*

Abstract: Previous studies with maleylacetone *cis-trans*-isomerase suggest that the coenzyme, glutathione (GSH), may act as a nucleophile in the enzyme-catalyzed cis-trans isomerization. In the absence of enzyme, reaction of the coenzyme with maleylacetone or its trans isomer, fumarylacetone, has been found to lead to an adduct, *S*-2-(4,6-dioxoheptanoic acid)glutathione. The same diastereomer of the adduct is formed from either geometric isomer, suggesting that the reaction of GSH with maleylacetone proceeds by a slow catalyzed cis-trans isomerization followed by a rapid reaction of GSH conjugate addition to the trans isomer. This mechanism is confirmed by an isotope dilution experiment and a kinetic method involving repetitive addition of fractional equivalents of GSH to a solution of maleylacetone. The intermediate formed during GSH-catalyzed cis-trans isomerization is suggested to be the dienediol formed by sulfur attack at C-2, and this intermediate undergoes bond rotation and elimination before protonation at C-3 can take place. By analogy and additional evidence, a similar mechanism and intermediate are suggested for the enzyme-coenzyme-substrate reaction.

Aromatic amino acids such as tyrosine and phenylalanine are metabolized in mammalian liver to a common intermediate, homogentisic acid (2,5-dihydroxyphenylacetic acid). Oxidative cleavage of the aromatic ring yields maleylacetoacetate (Ia), which in turn undergoes enzyme-catalyzed cis-trans isomerization to fumarylacetoacetate (IIa, eq 1).[2] Enzymes capable of carrying out these same transformations are also found in bacteria such as *Vibrio 01*.[3] The focus of attention in this paper is concerned with the mechanism of the catalyzed cis-trans isomerization reaction.



Ia, R = CO_2^-
b, R = H
II

(1)

The mechanistic investigation is simplified to some degree by the finding that maleylacetone (Ib) is also rapidly isomerized by both the liver and bacterial *cis-trans*-isomerase. Action of the enzyme, isolated from either of these two types of sources, requires the presence of the tripeptide, glutathione (γ-Glu-Cys-Gly, henceforth GSH), as a coenzyme. Studies concerned with the mechanism of the reaction and the role of enzyme and coenzyme have been actively pursued in our laboratory.[4]

Enzyme-catalyzed reactions, requiring a coenzyme, often bear strong mechanistic similarity to the reaction of the same substrate and coenzyme when the enzyme is absent. Hence it was of interest to investigate some aspects of the substrate-coenzyme reaction to see what might be learned from it about the enzyme-catalyzed reaction. In this paper we show that the coenzyme itself catalyzes the cis-trans isomerization of maleylacetone, and this reaction exhibits similar properties to the enzymatic reaction.

In a previous study it was shown that GSH binds to the enzyme, maleylacetone *cis-trans*-isomerase, isolated from *Vibrio 01*, through the backbone of the tripeptide and that the coenzyme's sulfhydryl points away from the enzyme surface. Hence the sulfur is available to interact with substrate. This could be seen from the fact that S-alkylated analogues of glutathione are powerful, reversible competitive inhibitors for GSH. Moreover, glutathione bound through its sulfur to an alkyl chain spacer arm, which in turn is bound to agarose, serves as an efficient adsorbent for affinity chromatography of the enzyme.[4a] Kinetic studies of enzyme-catalyzed cis-trans isomerization in the presence and absence of glutathione-analogue competitive inhibitors reveal that a new GSH molecule associates with enzyme for each turnover of substrate, suggesting that GSH reacts with substrate during the isomerization process. These studies also show that the *cis-trans*-isomerase from *Vibrio 01* has a preferred binding order: maleylacetone on first followed by glutathione.

Experimental Section

^{1}H NMR spectra were obtained at 100 MHz on a Jeol JNM-MH-100 and at 360 MHz on a Bruker WH-360. Ultraviolet spectra were obtained on a Cary 14.

***S*-2-(4,6-Dioxoheptanoic acid)glutathione (III) from 4,6-Dioxo-*cis*-2-heptenoic Acid.** 4-Acetonylidenebut-2-en-4-olide[5] (*Z*) (0.571 g) was added to 10 mL of 1 N KOH, which was cooled in ice. The mixture was stirred until the solid dissolved, and then the solution was neutralized with 1 N HCl. Glutathione (1.27 g, reduced form, Sigma Chemical Co.) was dissolved in 5 mL of water, and the pH was adjusted to neutrality by adding 1 N KOH. The two solutions were combined and the reaction was complete in minutes, as shown by the rapid loss of the vinyl NMR resonance. The solution was lyophilized and the reaction mixture chromatographed on a Dowex 1-X2 column (2.8 × 50 cm). The resin was converted previously to the formate form by washing it with a 2 M solution of formic acid followed by Millipore Q-purified water until the eluate was neutral. After loading the column with the reaction mixture, separation was accomplished with a water (1 L)–2 M formic acid (1 L) linear gradient, which is a variant of the method reported by Furano.[6] Derivatives of GSH were detected with ninhydrin. The fractions from 1230 to 1540 mL containing the desired product were combined and the solvent was removed under reduced pressure and room temperature; yield 1.507 g. A portion of this (0.390 g) was rechromatographed on a second column (1.5 × 61 cm) of Dowex 1-X2 in its formate form using the same gradient as above. The fractions containing the product were concentrated and the remaining solution was lyophilized to yield a white powder: UV λ_{max} (0.03 M potassium phosphate buffer, pH 5.0) 275 nm (log λ 3.33).

A small sample (17 mg) was further purified in several batches by high-pressure liquid chromatography (LC) on a preparative reverse phase column (LiChrosorb RP-18, 10 μm) using a solution of 33% methanol–67% 0.01 M acetic acid in water as the eluent. Fractions containing the desired compound were concentrated under reduced pressure at 30 °C, combined, and lyophilized. The solid obtained was dissolved in H_2O and lyophilized. This process was repeated twice to remove traces of acetic acid. Anal. Calcd for $C_{17}H_{25}O_{10}N_3S\cdot 2H_2O$: C, 40.88; H, 5.85; N, 8.41. Found: C, 40.95, H, 5.48; N, 8.50.

***S*-2-(4,6-Dioxoheptanoic acid)glutathione from Fumarylacetone (4,6-Dioxo-*trans*-2-heptenoic Acid).** Fumarylacetone was prepared as previously described.[5] Neutral solutions of 67 mg of fumarylacetone

0002-7863/79/1501-3091$01.00/0

in 2 mL of water and 136.5 mg of glutathione in 2 mL of water were mixed. After a few minutes the solution was acidified with 1 N HCl to pH 2, and the reaction mixture was concentrated under reduced pressure. The product mixture was purified on a Dowex 1-X2 column as described above to yield a white solid: NMR in D_2O (pH 1.5), internal DSS, δ 2.30 (t, 2 H, β-CH_2-Glu), 2.28 (S, 3 H, methyl), 2.65 (t, γ-CH_2-Glu), 2.85–3.5 (m, 4 H, β-CH_2-Cys, β-CH_2-fumaryl), 3.84 (t, 1 H, α-CH-Glu), 4.06 (s, 2 H, CH_2-Gly), 4.16 (t, 1 H, α-CH-fumaryl), 4.6 (t, α-CH-Cys partially under HDO peak); UV λ_{max} (0.03 M potassium phosphate buffer, pH 5.0) 275 nm (log ϵ 3.34).

Conjugate Addition of GSD to Maleylacetone in D_2O. 4-Acetonylidenebut-2-en-4-olide (*Z*) (31.70 mg, 0.230 mmol) was hydrolyzed in D_2O in the usual way.[5] An equivalent amount of GSH (70.72 mg, 0.230 mmol) in D_2O was neutralized to pD 8.9. The two solutions were mixed and the resulting solution had a pD of 8.4 in a total volume of 0.7 mL. ^1H NMR spectra at 100 MHz were recorded within a few minutes of mixing. After the vinyl resonances vanished, the pD was adjusted to pD 3.6 and NMR spectra were again recorded.

Kinetic Studies. The rate of reaction of GSH with maleylacetone or fumarylacetone was studied under pseudo-first-order conditions where GSH was in excess. Solutions of GSH in 0.07 M phosphate buffer (pH 7.16) were prepared and added to a 1- or 10-cm quartz cell. The reaction was initiated by adding a small amount of maleylacetone or fumarylacetone, in phosphate buffer, to the cell with the aid of a micropipet. The cell was shaken and returned quickly to the thermostated sample compartment of a Cary 14 spectrophotometer. The loss of optical density of 280.5 nm with time was recorded.

In other studies the conversion of the adducts to longer wavelength absorbing material was studied. *S*-2-(4,6-Dioxoheptanoic acid)glutathione (1×10^{-4} M) in 4×10^{-3} M potassium phosphate buffer (pH 7.4) was examined for the appearance of absorption at 318 nm with time. Rate measurements were continued for 10 half-lives.

Initial rate measurements of the appearance of the 318-nm absorption from III in aqueous solution at pH 7.4 in the presence of active maleylacetone *cis-trans*-isomerase were also carried out.

Kinetic studies were also carried out by using LC to monitor the concentration of IIb. In a typical run a 0.045 M solution of maleylacetone in 0.075 M phosphate (pH 7.2) was prepared in a total volume of 1.65 mL. Similarly a solution of GSH (0.0686 M) in 0.07 M phosphate buffer (pH 7.2) was prepared in 1 mL. At specific times, noted in the Results section, 100-μL samples of the GSH solution were added to the whole maleylacetone solution and immediately thereafter a 100-μL aliquot was withdrawn and quenched with 10 μL of 3 N HCl within 10 to 20 s after the GSH addition. The quenched sample (90 μL) was injected quickly onto a preparative reverse phase column (LiChrosorb RP-18, 10 μm, 0.68 × 46 cm) and eluted with a 45% methanol–55% 0.05 M acetic acid (in water) solution. The area of the fumarylacetone peak, detected at 254 nm with an Altex UV flow detector (Model 153), was determined by the cut-and-weigh procedure and was calibrated by using known concentrations of pure fumarylacetone in the same quenched buffer solution.

In a few instances the very slow rate of autoisomerization of maleylacetone was also determined by LC on a reverse phase column as described above.

The Nature of the 318-nm Material. Equal aliquots of a solution of III were transferred to ampules and each was purged of oxygen by carrying out several freeze–pump–thaw cycles on a high vacuum line. The ampules were then sealed and the substrate was allowed to undergo reaction at 25 °C to produce the 318-nm compound. After 48 h the ampules were either opened and assayed immediately or heated for 10 min at 100 °C prior to opening. The contents were assayed for free thiol with Ellman's reagent[7] and examined by LC on a Zipax SAX (DuPont) column under conditions (0.01 M sodium nitrate) capable of separating maleylacetone, fumarylacetone, and the glutathione adduct of these compounds.

LC Product Analysis of the Enzymatic Reaction. In studies requiring the absence of mercaptoethanol (used to stabilize the enzyme against oxidation), the enzyme was either dialyzed against a 0.01 M potassium phosphate buffer containing 4×10^{-5} M GSH, or a phosphate buffer in an argon atmosphere when GSH was absent. In the latter experiments dialysis was carried out in an all glass bubbling tower having a fine glass frit. Argon was passed through the buffer for 30 min prior to the introduction of the enzyme. The enzyme, in its dialysis bag, was added quickly and the argon sweep continued. The enzyme was dialyzed in this way against two batches of 250 mL of buffer for about 2 h each. The final mercaptoethanol concentration is expected to be less than 1.4×10^{-8} M.

Aliquots of this enzyme were mixed with maleylacetone at pH 7.4 under argon and aliquots of the mixture were withdrawn periodically and injected onto a 0.2 × 60 cm Zipax SAX column. Elution was accomplished with a 0.01 M sodium nitrate–0.01 M sodium bicarbonate solution adjusted to pH 7.3 with nitric acid. Detection was made at 254 and 313 nm by an Altex UV monitor. Peak areas were calculated by the cut-and-weigh technique.

Enzymatic Isomerization of Maleylacetone in D_2O. 4-Acetonylidenebut-2-en-4-olide (7.42 mg) was dissolved in 0.2 mL of cold 1 N potassium deuteroxide. When the solid dissolved, the solution was neutralized with 0.2 mL of 1 N deuterium chloride and then diluted to 3 mL with 0.03 M potassium phosphate buffer in D_2O. The pD was adjusted to 7.8.

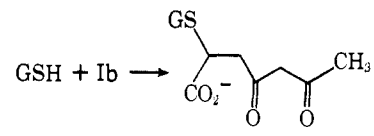
A solution of the isomerase (1.0 mL, 1 unit/mL) was mixed with 0.6 mL of a 4.82 mM solution of GSH in 30 mM potassium phosphate buffer in D_2O (pD 7.8). The enzyme had been previously dialyzed against 30 mM phosphate buffer in D_2O. The enzyme–GSH solution was added to the above maleylacetone solution in 0.4-mL aliquots at 10-min intervals while the extent of reaction was followed spectrophotometrically. After about 1 h the solution was acidified with 1 N HCl to a pD of 2.7 and the solution was extracted four times with 2-mL aliquots of ether. The combined ether extracts were dried over anhydrous sodium sulfate, and the solvent was evaporated to yield 3.3 mg (43%) of fumarylacetone. The ^1H NMR spectrum at 100 MHz was examined.

Maleylacetone-*2,3*-^{14}C. Maleic anhydride-*2,3*-^{14}C (Merck, Lot No. 58 CD 478, 0.1 mCi, sp act. 1.15 mCi/mmol) was mixed with 5 g of maleic anhydride, and the mixture was distilled at atmospheric pressure. The remainder of the synthesis is as described previously for the preparation of 4-acetonylidenebut-2-en-4-olide.[5] The first crop of twice recrystallized material was collected and amounted to 60 mg. A small aliquot was counted in a Beckmann scintillation counter using Aquasol as the solvent.

Isotope Dilution Experiments. A typical procedure is as follows. A neutralized solution of fumarylacetone (0.100 M, 0.065 mL) was mixed with a neutralized solution of maleylacetone-*2,3*-^{14}C (0.0261 M, 0.65 mL) containing 0.076 M potassium phosphate buffer. A 9.1×10^{-2} M neutralized solution of GSH (0.065 mL) was added to this mixture. After 3 min the whole reaction mixture was injected onto the preparative phase column and the components were eluted with a solution of 30% methanol–70% 0.05 M acetic acid (v/v) with a flow rate of 2.5 mL/min. Fumarylacetone eluted at 27 min and was collected over a volume of 108 mL. The solvent was removed at reduced pressure. The residue was dissolved in methanol, and the solvent removed again at reduced pressure. This was repeated once more to remove all traces of acetic acid. The dry residue was dissolved in 2.0 mL of methanol. An aliquot was taken and further diluted until an optical density at 311 nm could readily be measured. The remaining material was counted. In this way the specific activity (cpm/mmol) was determined.

Results

The Reactions of Maleylacetone and Fumarylacetone with Glutathione in the Absence of Isomerase. The NMR and UV spectra of maleylacetone (Ib) and fumarylacetone (IIb) in aqueous solution have been reported previously.[5] Equal concentrations of Ib or IIb and GSH were allowed to react at near pH 7. During reaction the optical density at 314 nm, due to Ib or IIb, as well as the ^1H NMR of the vinyl protons at C-2 and C-3 decrease, suggesting conjugate addition at these carbons (eq 2). The product mixture from each reaction was chromatographed on Dowex 1-X2 yielding a single product from each reaction. The products when compared to each other and as a mixture by analytical LC on Zipax SAX, LiChrosorb RP-18, and AS Pellionex SAX columns indicated identical properties.



(2a)

III

GSH + IIb ⟶ III (2b)

Table I. ^1H NMR Chemical Shifts of III vs. pD

	pD						
	1.91	2.58	3.45	3.97	4.39	4.77	5.41
new triplet	3.84	3.86	3.78	3.75	3.73	3.70	3.68
Glu-α-CH	4.16	4.09	3.88	3.86	3.86	3.86	3.84
Gly-CH_2	4.06	4.08	4.05	3.96	3.91	3.86	3.83
Glu-γ-CH_2	2.65	2.64	2.58	2.58	2.59	2.58	2.58
Glu-β-CH_2	2.30	2.30	2.26	2.23	2.24	2.24	2.24
CH_3	2.28	2.30	2.29	2.29	2.30	2.30	2.29

Table II. Rate Constants and Activation Parameters for the Disappearance of Maleylacetone (Ib) and Fumarylacetone (IIb) in the Presence of GSH[f]

	k_2, M^{-1} s^{-1}		$\Delta H^{\ddagger}$,	
	1.8 °C	23.25 °C	kcal/mol	$\Delta S^{\ddagger}$, eu
Ib + GSH[a]	0.0209[b]	0.248[c]	18.1	−0.1
IIb + GSH[a]	4.06[d]	11.0[e]	6.9	−30.2

[a] GSH concentrations were: [b] 1.55×10^{-2} to 1.97×10^{-2} M, [c] 3.08×10^{-3} to 4.03×10^{-3} M, [d] 2.29×10^{-4} to 2.40×10^{-4} M, and [e] 9.55×10^{-5} to 1.37×10^{-4} M. [f] In the presence of 0.07 M potassium phosphate buffer (pH 7.16).

The product has its maximum at 275 nm. The ^1H NMR spectrum of III shows a new triplet (1 H) and multiplet (2 H) when compared to the corresponding spectrum of GSH (both in D_2O). The new triplet is undoubtedly due to the proton on carbon bearing the GS moiety and the multiplet due to the protons on the vicinal carbon. To show that sulfur is bonded to C-2 of the adduct as shown for III rather than bonded to C-3, ^1H chemical shifts vs. pD were examined. These are given in Table I. The $\Delta\delta$ for the new triplet, over the acidity range where a nearby carboxyl group is dissociating, is almost as large as is observed for the α protons of the glycyl residue in GSH itself. It is considerably larger than the $\Delta\delta$ for the β-CH_2 of the glutamyl residue. The pD for half the change in chemical shift of the new triplet occurs at about 4.0. These observations together with elemental analysis and UV spectra are consistent with the product of addition having the structure shown for III instead of the isomer where the glutathiyl sulfur is bonded to C-3. Unfortunately the corresponding new multiplet, due to the protons at C-3, overlaps with the resonances due to Cys-β-CH_2 and prevents us from locating the center of the resonances at all pDs examined. At some pDs measured, however, a prominent part of the multiplet could be discerned, and over the pD range 1.9 to 5.0 this peak moves only 0.02 ppm upfield.

The reaction of Ib with GSH to yield III was carried out in D_2O to investigate the extent of deuterium incorporation. The reaction was carried out in an NMR tube with equivalent amounts of GSH and Ib at a pD of 8.4. After the vinyl proton resonances disappeared, the pD was adjusted to 3.6 and spectra and integrals were recorded. The spectrum is essentially the same as that for III formed in H_2O except for the resonance located between the Glu-γ-CH_2 and the overlapping resonances due to Gly-CH_2, Glu-α-CH, and the new triplet. This resonance located at about δ 3.16 is broad and is due to the Cys-β-CH_2 plus the resonance at C-3. This broad peak integrates for 3.09 protons for III formed in D_2O and 4.0 protons when formed in H_2O; the Glu-γ-CH_2 protons of III, formed in either solvent, serve as an internal standard. Integration errors are anticipated to be about 5%.

The rate of reaction of GSH with Ib and IIb in buffered aqueous solution at pH 7 was examined. In each case the kinetics were studied with excess GSH so that the rate was pseudo first order. Kinetics were measured for the loss of Ib and IIb at 280.5 nm, an isosbestic point in the very slow equilibration of III with IV, a subsequent product (vide infra). In this way, only the first step (eq 2a or 2b) is detected kinetically. Second-order rate constants for eq 2a and 2b at two different temperatures are given in Table II. Each is the result of at least two determinations. Good first-order kinetics were observed for at least 80% loss of either substrate. Activation parameters based on the measured rate constants are also included in the table.

As mentioned above, the adduct III which has its UV maximum at 275 nm undergoes further transformation to a compound having its maximum at 318 nm. This new compound (IV), although it absorbs in almost the same place as fumarylacetone or maleylacetone, exhibits no vinyl proton resonance. Moreover, on analytical LC using a strong anion exchange column, Zipax SAX, IV elutes considerably later than fumarylacetone or maleylacetone when dilute aqueous sodium nitrate is the eluting solvent. These results suggest that IV has a higher net negative charge than either Ib or IIb. It was of interest to see whether the conversion of III → IV resulted in the release of GSH or oxidized glutathione (GSSG). These were tested for by Ellman's reagent[7a] and by the glutathione reductase assay,[7b] respectively. Both of these were negative. IV, formed in solution but not isolated, when heated at 100 °C for about 10 min in vacuo, however, releases GSH and fumarylacetone as shown by a positive test for free thiol (Ellman's reagent) and by LC, respectively. These results suggest that the conversion of III to IV does not lead to the separation of a glutathiyl residue from a Ib or IIb adduct. We speculate that the development of longer wavelength absorption in the III → IV reaction may be due to formation of an intramolecular Schiff's base. Because this appears to be a side reaction, unimportant to the main question of the mechanism of isomerization, the structure of IV was not investigated further. The kinetics of its formation, however, were determined because of its possible effect on the calculation of the rate constant for the Ib or IIb reaction with GSH. The rate of the single step, III → IV, in phosphate-buffered solution was determined by measuring the appearance of absorption at 318 nm. At pH 7.4 the average first-order rate constant at 22 °C is 8.85×10^{-5} s^{-1}, which is about a factor of ten smaller than the pseudo-first-order rate constant measured for the formation of III from Ib.

Adduct Formation from the Cis Isomer. Because the question of the mechanism of adduct III formation from GSH and the cis isomer Ib could not be resolved by standard methods, two different types of experiments were used to elucidate the sequence of events: (1) isotope dilution and (2) method of repetitive GSH additions. These experiments were designed to answer the question: Is the trans isomer IIb an intermediate in the conversion of Ib to III? In other words, does a sequential (eq 3) or a parallel (eq 4) process operate?

$$\text{Ib} + \text{GSH} \rightarrow \text{IIb} + \text{GSH} \quad (3a)$$

$$\text{IIb} + \text{GSH} \rightarrow \text{III} \quad (3b)$$

$$\text{Ib} + \text{GSH} \rightarrow \text{III} \quad (4a)$$

$$\text{IIb} + \text{GSH} \rightarrow \text{III} \quad (4b)$$

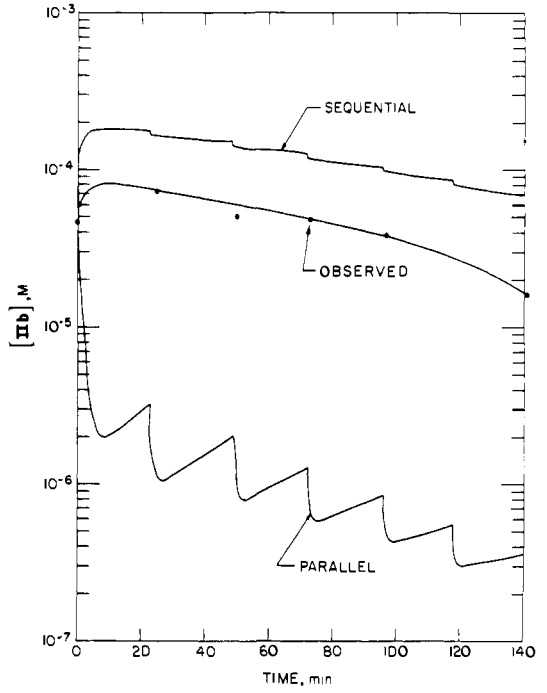


Figure 1. Concentration of fumarylacetone (IIb) vs. time. Maleylacetone (Ib) (44.6 mM, 1.65 mL, pH 7.15) was allowed to react with 100-μL aliquots of a 68.6 mM GSH solution. After each addition, 100-μL aliquots of solution were withdrawn, acidified, and analyzed for IIb by LC. Observed concentrations of IIb: solid circles. Computations of concentrations of IIb were carried out as described in the text. Each drop of IIb in the computer curves is the result of a GSH addition. The rises in IIb most obvious in the parallel mechanism curve are due to autoisomerization.

Isotope Dilution. Ib-^{14}C was synthesized and mixed with unlabeled IIb. A quantity of GSH, insufficient to react with all of the IIb present, was added. In a typical experiment [GSH]/[IIb] ≃ 0.75. The unreacted IIb was isolated by rapid LC and its specific activity measured. If a parallel sequence operates, the isolated unreacted trans isomer would be expected to have zero ^{14}C activity. If, however, a sequential process operates, the unreacted IIb that remains should have ^{14}C activity. These expectations must be modified because there is a complication of a very slow simultaneous autoisomerization (i.e., noncatalytic) of Ib to IIb. In the absence of GSH, Ib in aqueous phosphate buffer (pH 7.2) is converted to IIb with first-order rate constants of 1.66×10^{-5} and 5.43×10^{-7} s^{-1} at 23.2 and 0 °C, respectively. Moreover, because of this slow autoisomerization process, reactant Ib, which is obtained by preliminary hydrolysis of 4-acetonylidinebut-2-en-4-olide, is always contaminated with a small amount of IIb. In our hands this amounts to 0.1% IIb. Attempts to remove this small amount of contaminant have resulted in the formation of a larger fraction of IIb.

The concentration of IIb-^{14}C generated by either mechanism (eq 3 or 4) when GSH is essentially consumed can be calculated from the known rate constants for (a) the disappearance of *cis*-(Ib + GSH), (b) the disappearance of *trans*-(IIb + GSH), and (c) the autoisomerization (Ib → IIb) and the initial quantity of IIb-^{14}C present in reactant Ib-^{14}C at $t = 0$. The concentrations of IIb-^{14}C and hence the specific activities by each mechanism were computed by numerical integration using 10^4 intervals. The results shown in Table III are in good agreement with a sequential mechanism operating.

Method of Repetitive GSH Additions. Another type of experiment to determine whether a parallel or sequential mechanism operates was carried out. Near 0 °C the rate of disappearance of the trans isomer in the presence of GSH is about 200 times faster than the rate of disappearance of the cis isomer at the same GSH concentration. If these relative rate constants are to represent those for eq 4b and 4a (parallel), respectively, then repetitive additions of GSH to a solution containing Ib, with a small amount of IIb present, will result in the depletion of the IIb concentration. This expectation rests upon the condition that GSH additions and subsequent analysis are rapid with respect to autoisomerization.

Table III. Specific Activities of Fumarylacetone (IIb) Isolated from Reaction Mixtures Initially Containing GSH, Maleylacetone-*2,3*-^{14}C (Ib-^{14}C), and Fumarylacetone

	sp act., cpm/mmol			
		IIb		
			calcd[c]	
run	Ib-^{14}C	obsd	sequential[d] mechanism	parallel[e] mechanism
A[a]	1.24×10^6	3.09×10^5	2.67×10^5	4.69×10^4
B[b]	1.24×10^6	2.0×10^5	1.70×10^5	7.41×10^4

[a] Initial conditions: [Ib-^{14}C] = 2.12×10^{-2} M, [IIb] = 8.49×10^{-3} M, [GSH] = 7.39×10^{-3} M, [IIb-^{14}C] = 1.06×10^{-4} M. Reaction time = 3.0 min at 23.2 °C. [b] Initial conditions: [Ib-^{14}C] = 2.48×10^{-2} M, [IIb] = 1.26×10^{-2} M, [GSH] = 8.83×10^{-3} M, [IIb-^{14}C] = 1.24×10^{-4} M. Reaction time = 10.0 min at 23.2 °C. [c] Computed by using the rate constants given in Table II and text. [d] Ib + GSH → IIb + GSH; IIb + GSH → III. [e] Ib + GSH → III; IIb + GSH → III.

A different prediction results from assuming that the rate constants for the disappearance of Ib and IIb are for the steps 3a and 3b as in the sequential mechanism. It can be easily shown that the ratio of [Ib]/[IIb] ≃ k_{3b}/k_{3a} = 200 during reaction with GSH. If only fractional equivalents of GSH are added repetitively, then [Ib] does not decline rapidly and hence [IIb] is also relatively constant. Again, this is true provided that GSH additions and product analysis are rapid with respect to autoisomerization. In any case, just as in the isotope dilution experiment, here, too, the time-dependent concentrations of IIb can be calculated, assuming this mechanism, from the known rate constants and the initial quantity of IIb present.

Experimentally, 0.1 equiv of GSH is repeatedly added to a buffered solution of Ib, and immediately after each addition an aliquot of the solution is rapidly acidified and injected onto a preparative reverse phase column. IIb is detected at 254 nm with a flow UV detector and quantitated by measuring the area of the peak. In Figure 1 are shown the computer [IIb] vs. time curves for the sequential and parallel mechanisms and the observed curve. These are presented as semi-log plots because of the large range of concentrations encountered. There are two significant features in the figure. (a) The observed [IIb] curve lies about a factor of two away from that computed for the sequential mechanism but a factor of 50 away from the parallel mechanism. (b) The observed curve shows an initial increase in the concentration of IIb with time. A similar initial increase is seen for the curve calculated on the basis of a sequential mechanism. This is because the [Ib]/[IIb] at $t = 0$ is greater than 200, and this ratio of concentrations must be established during reaction. This takes place by the generation of a higher IIb concentration. The parallel mechanism on the other hand predicts an initial decrease in [IIb]. Agreement with the sequential mechanism is very good, considering that the computed curves depend on three separately determined rate constants.

Studies with the Isomerase. It was of interest to see whether III might be a substrate for the isomerase and thereby be converted to IIb and GSH. Since IIb absorbs at 314 nm its formation could in principle be detected by monitoring the optical density of a solution containing isomerase, III, and GSH. The conversion of III to IV contributes to the absorption,

Table IV. Product Analysis during Enzyme-Catalyzed Isomerization of Maleylacetone[a]

time, min	[Ib], M	10^4 [IIb], M	([Ib] + [IIb])/[Ib]$_0$
0	4.0	0	1.0
0.75	2.76	0.965	0.93
2	1.81	1.88	0.92
3	1.62	2.35	0.99
4	1.45	2.56	1.0
5	1.27	2.61	0.97
10	1.05	2.85	0.98
13	1.05	2.84	0.97
20	1.02	2.95	0.99

[a] The enzyme was previously dialyzed against 2×10^{-4} M GSH in an argon atmosphere in 0.01 M phosphate buffer (pH 7.4) to remove mercaptoethanol. The substrate was added. The concentration of GSH during reaction was 4×10^{-5} M. Analysis was performed by LC on a Zipax SAX column using 0.01 M $NaNO_3$–0.1 M $NaHCO_3$ eluting solvent.

but as shown above this reaction is very slow ($t_{1/2}$ = 130 min). Initial rates of optical density increase of a solution containing III in the presence and absence of isomerase and GSH were found to be the same within experimental error, indicating that III is not a substrate for the isomerase.

Material Balance in the Enzymatic Reaction. The material balance in the reaction shown in eq 1 was examined. The enzymatic reaction was carried out in the presence of a relatively low GSH concentration to reduce the rate of the formation of III to an insignificant amount. At 4×10^{-5} M GSH, the enzyme is only about 20% saturated with coenzyme, but a fast enzymatic rate can be achieved by using a large amount of enzyme. After mixing isomerase, GSH, and Ib, aliquots at various reaction times were assayed for Ib and IIb by analytical LC. The results are shown in Table IV. The *cis-trans*-isomerase is very labile to oxygen and consequently stabilized with mercaptoethanol. For the purposes of this experiment it was necessary to remove mercaptoethanol and, as can be seen from the table, activity is unfortunately lost after about 5 min. The results shown in the last column, however, clearly show that there is no evidence for the formation of a substantial amount of a relatively stable intermediate and that the product of the reaction is indeed the trans product.

Enzymatic Reaction in D_2O. Isomerization of Ib to IIb catalyzed by the isomerase was carried out in D_2O to determine the extent of vinyl proton exchange at C-2 and C-3. The NMR of these protons is shown in Figure 2 along with that observed for the trans product formed in H_2O. As can be seen both AB quartets appear the same indicating little (<5%), if any, proton exchange at C-2 and C-3 during reaction in D_2O.

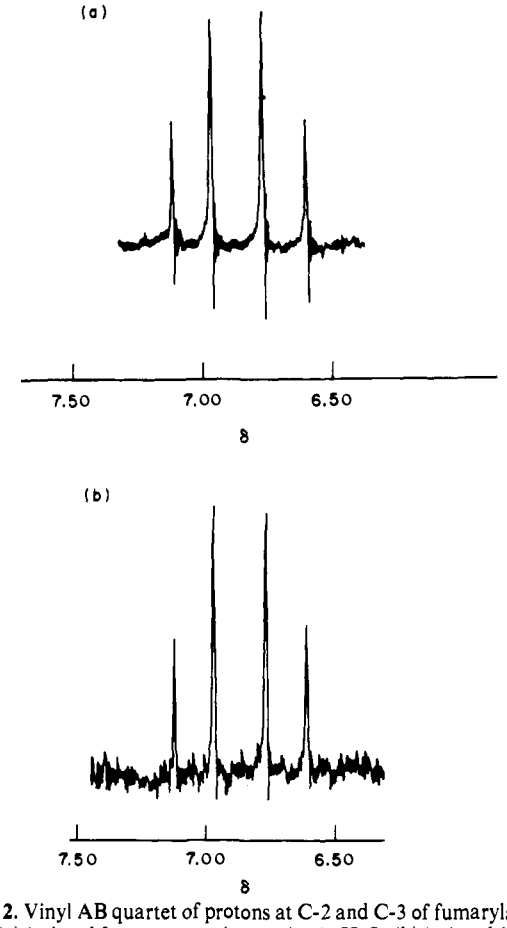


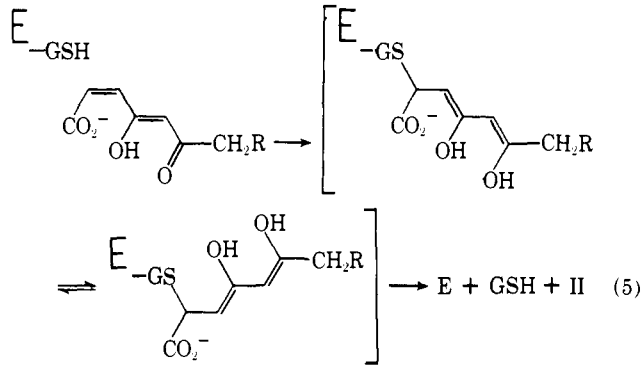
Figure 2. Vinyl AB quartet of protons at C-2 and C-3 of fumarylacetone (IIb): (a) isolated from enzymatic reaction in H_2O; (b) isolated from enzymatic reaction in D_2O.

Discussion

Thiocyanate ion was shown previously to catalyze the cis-trans isomerization of maleic acid in aqueous acid.[8] Thiocyanate, a good nucleophile, is a more effective catalyst than chloride, and perchlorate shows no catalytic activity. Consequently the mechanism of the catalyzed reaction was postulated to involve nucleophilic attack on the olefinic carbon of the undissociated acid to provide an intermediate capable of facile internal rotation about the C-2–C-3 bond. That a proton is not also added to olefinic carbon is shown by the lack of vinyl proton exchange with solvent during isomerization.[9,10] This postulated intermediate was not detected until secondary α-deuterium kinetic isotope effects were employed to probe the nature of the transition state. Maleic-*2,3-d* acid when catalytically isomerized by thiocyanate ion exhibits an inverse isotope effect, $k_H/k_D = 0.89$ (80 °C).[9] The isotope effect result is consistent with the mechanism previously postulated.

Maleylacetone (Ib) also undergoes rapid thiocyanate-catalyzed cis-trans isomerization and when carried out in D_2O leads to no exchange of protons at C-2 and C-3.[11] The mechanism is believed to be similar: nucleophilic addition to C-2 to yield an intermediate capable of internal rotation about the C-2–C-3 bond and subsequent loss of thiocyanate.

As mentioned in the introductory statement, previous experiments have shown that GSH, bound to enzyme, has its sulfhydryl pointing away from the enzyme surface and therefore is most likely available for nucleophilic attack on the substrate in a mechanism of the type shown in eq 5, which is



(5)

similar to the thiocyanate-catalyzed reactions.[12] It should be emphasized here that the direct product of the enzymatic reaction is the trans isomer and not an adduct of GSH-conjugate addition to the substrate. Equilibrium between cis and trans isomers lies far over toward trans.[4b,13] As seen in Table IV, rapid LC analysis shows very good material balance in the sum of cis and trans isomers and leaves little possibility for the si-

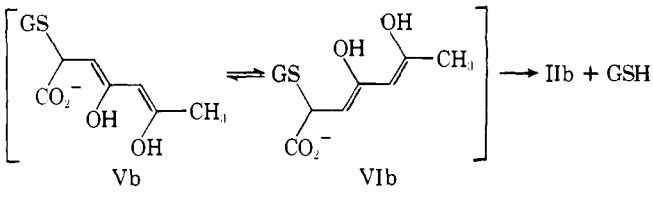
multaneous enzyme-catalyzed formation of an adduct.[14] Moreover, adduct, formed nonenzymatically, is not acted upon by the enzyme (vide infra). Certain features of this mechanism (eq 5), such as the site of attack and the nature of the intermediate, are suggested by drawing analogy both to the reactions discussed above and the reaction of substrate and coenzyme in the absence of isomerase which is discussed below.

In the absence of enzyme, GSH adds conjugatively to the C-2–C-3 double bonds of both maleylacetone and fumarylacetone. The adducts from each reaction have been shown to be identical by LC on three different columns and by ^{1}H NMR at 360 MHz. The assigned structure (III) is in accord with elemental analysis, UV, and NMR data. Moreover, the site of attachment of sulfur at C-2 has been assigned on the basis of ^{1}H chemical-shift data vs. pD measurements.

The kinetics of each reaction have been studied at pH 7 at each of two temperatures under pseudo-first-order conditions. The bimolecular rate constants are listed in Table II. GSH reacts with the trans isomer very much faster than with the cis isomer. In the GSH addition no intermediate is detectable by NMR or UV. That is to say that starting with the cis isomer (Ib) in D_2O one can only detect the disappearance of the cis vinyl AB quartet during reaction with GSH without the transitory appearance of any other vinyl resonance. This suggests that the reaction of Ib with GSH is simply conjugate addition (eq 2a). When contrasted with the reaction of Ib + GSH in the presence of isomerase (eq 1), however, this suggestion raises a question, for it appears to confer special properties on the isomerase which in the enzymatic reaction directs the substrate to undergo isomerization but not GSH addition. (Of course along with the enzymatic reaction, conjugate addition of GSH to the substrate and product take place in a nonenzymatic side reaction. The enzyme requires a very low concentration of GSH to activate it ($K_M = 1.4 \times 10^{-4}$ M) and hence these nonenzymatic side reactions are much slower than the enzyme-catalyzed isomerization.)

In the reaction of Ib or IIb with GSH, a new chiral center at C-2 is generated. Since GSH is itself a single diastereomer of γ-Glu-Cys-Gly, its reaction with Ib and IIb is expected to exhibit stereospecificity and lead to a different diastereomeric ratio of adducts from the two substrates. Only a single adduct, however, can be detected from each reaction and they are identical. One way to explain this paradox is to suggest that the reaction of Ib + GSH is not one of direct addition but rather a catalyzed isomerization of IIb (eq 3a). If this is so then the rate constant for the disappearance of Ib in the presence of GSH (Table II) is for the first step in a sequential reaction mechanism (eq 3a and 3b) for the formation of adduct from Ib and GSH. Since k_{3a} is much smaller than k_{3b}, the detection of a small amount of IIb formed from Ib by conventional kinetic methods, NMR, or UV would be impossible. We therefore resorted to two other types of experiments, (a) isotope dilution and (b) repetitive GSH additions, to demonstrate that a sequential mechanism indeed operates when Ib and GSH react.

The course of the reaction being established, a question arises as to the details of the isomerization step. Does the GSH-catalyzed isomerization proceed through full conjugate addition to produce an unstable diastereomer of III (III′), which undergoes rapid elimination to yield IIb (i.e., eq 4a followed by the reverse of 4b), or is a dienediol intermediate (Vb) first generated and then undergoes internal rotation (Vb → VIb), and finally is GSH ejected before addition can take place at C-3 of Vb? The extent of deuterium incorporation as determined by NMR indicates that this latter path obtains. The reaction of Ib + GSH carried out in D_2O yields III with 0.91 atom of deuterium incorporated at C-3. Were an addition-elimination mechanism (eq 4a to yield a different diastereomer, reverse 4b, followed by formation of III) operating, one would expect 1.5 atoms of deuterium incorporated at C-3 if a primary intramolecular isotope effect in the elimination is neglected. It is more likely that such an isotope effect would operate and, in that case, close to two atoms of deuterium would be expected at C-3.



A similar path (eq 5) has already been suggested for the enzyme-catalyzed reaction, and if this suggestion is correct enzymatic isomerization of Ib to IIb in D_2O should also proceed without deuterium incorporation at C-3 in IIb. This experiment was carried out. NMR spectra of the C-2–C-3 vinyl region (Figure 2) show no evidence for D exchange and hence support the mechanism shown in eq 5. It should be pointed out, however, that after thiol addition to C-2 a group on the enzyme could conceivably transfer a proton to one face of Ib at C-3. Rotation could occur about C-2–C-3 while C-3 to -7 remain fixed in position at the active site. Elimination of GSH could then be accomplished by removal of the same proton at C-3 by the conjugate base of the enzymic proton donating group. In such a case elimination would proceed with apparent opposite stereochemistry to addition and result in no deuterium incorporation.

No adduct accumulates during enzymatic reaction. Were an enzymatic addition-elimination mechanism to operate, it might be anticipated that the enzyme would catalyze the elimination of GSH from the adduct to form IIb. As discussed above, however, even though two diastereomers of the adduct are possible only one forms and the possibility that this is a substrate for the enzyme was examined. III in the presence of enzyme does not yield IIb but very slowly converts to an isomer IV at the same rate as in the absence of the isomerase. Addition of GSH to that system has no effect. Thus support for the mechanism (eq 5) gained by the observation of no D exchange is strengthened by the finding that III is not a substrate for the enzyme.

Although the rate of the enzyme-coenzyme-catalyzed isomerization is 10^6-fold greater than the coenzyme-catalyzed isomerization, the details of that part of the mechanism involving the substrate-coenzyme interaction appear to be the same. Moreover, the mechanism encountered here is directly analogous to the thiocyanate-catalyzed cis-trans isomerizations of Ib and maleic acid. So the isomerase does not appear to alter the manner in which GSH reacts with substrate but rather increases the rate. How this is accomplished remains to be elucidated by further studies.

As shown above the reaction of the cis isomer Ib with GSH, in the absence of the enzyme, is isomerization, while that for the trans isomer IIb is conjugate addition. Both reactions appear to start out the same way, i.e., nucleophilic attack by the thiol group on C-2. In the former reaction the intermediate is stabilized for a sufficient time to allow internal rotation and expulsion of GSH before protonation occurs at C-3. In the latter reaction, however, proton addition occurs more rapidly. Why is there this remarkable difference in the rate of protonation at C-3? It is instructive to examine rates and activation parameters. The relative rates (k_{3a}/k_{3b}) are 1/45 at 22 °C and 1/200 at 2 °C, and the enthalpies and entropies of activation, as shown in Table II, are 18 kcal/mol and 0 eu for Ib (eq 3a) and 7 kcal/mol and −30 eu for IIb (eq 3b). We presume that the low activation energy and highly negative entropy of activation indicate that addition of sulfur to C-2 and a proton to C-3 of IIb occur concertedly. X-ray crystallography of GSH shows sulfur and α-Glu ammonium nitrogen only 5.6

Å away.[15] Dreiding models indicate that IIb could readily intercalate so that these groups are opposite C-2 and C-3, respectively. Assuming that the conformation of GSH in solution is similar to that in the crystal, the large negative $\Delta S_{IIb}^{\ddagger}$ may be due to the restriction of vibrational and rotational freedom of these groups in the transition state.[16] This path appears to be more difficult for the more angular Ib.

Acknowledgments. The authors thank Dr. W. S. Morrison for carrying out the experiment pertaining to the question of vinyl-proton exchange during enzymatic isomerization in D_2O. They would also like to thank Mr. Donald Lawler and Dr. Alan McLaughlin for the 360-MHz ^1H NMR spectra.

References and Notes

(1) Research was carried out at Brookhaven National Laboratory under contract with the U.S. Department of Energy and supported by its Office of Basic Energy Sciences and the National Institutes of Health, GM-18451.
(2) Lehninger, A. L. "Biochemistry", Worth Publishers: New York, 1970; p 442.
(3) Chapman, P. J.; Dagley, S. *J. Gen. Microbiol.* **1962**, *28*, 251.
(4) (a) Morrison, W. S.; Wong, G.; Seltzer, S. *Biochemistry* **1976**, *15*, 4228; (b) Seltzer, S. *J. Biol. Chem.* **1973**, *248*, 215.
(5) Fowler, J.; Seltzer, S. *J. Org. Chem.* **1970**, *35*, 3529.
(6) Furano, A. V. *Methods Enzymol.* **1971**, *17B*, 509.
(7) (a) Ellman, G. L. *Arch. Biochem. Biophys.* **1958**, *74*, 443; (b) Racker, E. *Methods Enzymol.* **1955**, *2*, 722.
(8) Nozaki, K.; Ogg, R. *J. Am. Chem. Soc.* **1941**, *63*, 2583.
(9) Seltzer, S. *Chem. Ind. (London)* **1959**, 1313.
(10) Horrex, C. *Trans. Faraday Soc.* **1937**, *33*, 570.
(11) Unpublished results of W. G. Frick.
(12) The mechanism shown is meant to focus on the reaction of coenzyme with substrate. It neglects, for the time being, any discussion of the interaction of substrate with enzyme and any activation that this interaction might provide. The intermediate here produced is shown protonated at O-6. The negative charge generated might be stabilized instead by a counter positive charge on the enzyme.
(13) Kisker, C. T.; Crandall, D. I. *Tetrahedron* **1963**, *19*, 701.
(14) See, however, Keen, J. H.; Jakoby, W. B. *J. Biol. Chem.* **1978**, *253*, 5654.
(15) Wright, W. B. *Acta Crystallogr.* **1958**, *11*, 632.
(16) Of course an intervening water molecule between C-3 and the ammonium nitrogen could serve as the proton donor were the solution nonbonded S–N distance to be larger than in the crystal. This would also contribute toward a large negative $\Delta S_{IIb}^{\ddagger}$.

Reagents for the Cross-Linking of Proteins by Equilibrium Transfer Alkylation

Sumita Mitra and Richard G. Lawton*

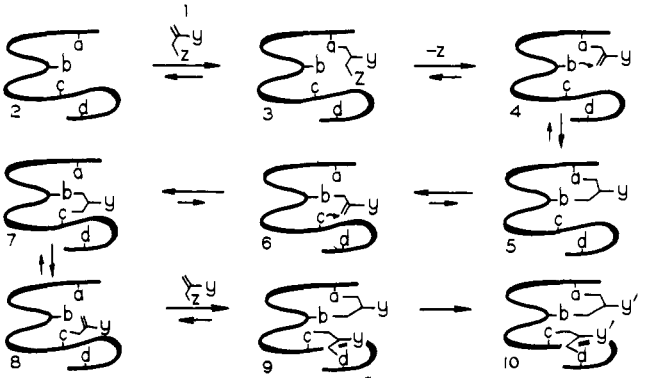
Contribution from the Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109. *Received November 27, 1978*

Abstract: Reagents (**11** and **12**) have been synthesized which can interact with nucleophilic groups on proteins and biopolymers by a sequence of consecutive Michael reactions yielding cross-linked and multi-cross-linked structures. Consecutive Michael reactions occur because Michael addition to the reagent then allows elimination of the trimethylammonium or mercaptonitrobenzoate function unmasking the latent double bond. A second Michael is then possible. These consecutive Michael reactions potentially allow the cross-link(s) to circumambulate the protein framework until the most thermodynamically stable cross-link equilibrium is established. Intermolecular cross-links can be established between protein subunits and multienzyme complexes. These reagents are designed so that addition and sequential cross-linking may be monitored by spectroscopy, and the cross-link may be subsequently fixed. This fixing process may be accomplished at variable times so as to provide a spectrum of cross-linked derivatives representing the sequence of steps. The chemistry of the process was established using cysteine, acetylcysteine, lysine, alkyl mercaptans, and alkylamines as models of protein residues. The intra- and intermolecular cross-linking character of these reagents was demonstrated using ribonuclease. Multiple cross-links could be introduced. In one of the modified ribonucleases, two links were introduced—one link was established between lysine residues 7 and 37; the other is probably between lysine residues 31 and 41. These links were determined by tryptic peptide mapping. A modified ribonuclease having three cross-links and intermolecularly cross-linked ribonuclease dimers and trimers were also produced.

Introduction

Chemical techniques for establishing the tertiary and subunit structures of enzymes, proteins, and biopolymers have traditionally depended upon cross-linking reagents with two essentially noninteracting alkylating or acylating groups.[1-5] Usually, chemical reagents used for the purpose of modification and cross-linking studies have not been differentiated from one another as to their ability to form equilibrium or nonequilibrium attachments during the reaction process.[6] Equilibrium cross-linking has been studied primarily within the framework of exchanges and recombinations of disulfide links of cystine residues,[7] and the concepts have not been extrapolated to reagent cross-linking. However, the nature of the bonding functions of most cross-links can be classified into two mechanistically distinct groups: kinetic (nonreversible) and equilibrium controlled reaction attachments. Our proposal is that these mechanistically different reagent characteristics can potentially alter the sites of residue interlinking. We wish to introduce compounds with generalized structure **1** (z = a leaving group, y = an electron-withdrawing substituent) having a unique association of functionality[7-11] which allows the study of equilibrium established cross-linked derivatives of proteins and biopolymers by consecutive Michael reactions.[12]

Scheme I



The process may be illustrated by considering the interaction of **1** with a diagrammatic protein **2** having several nucleophilic sites (a, b, c, etc., Scheme I). Michael addition[13-16] to an ex-

0002-7863/79/1501-3097$01.00/0