Maleylacetone *cis-trans*-Isomerase: Affinity Chromatography on Glutathione-Bound Sepharose. Two-Substrate-Binding Sequence from Inhibition Patterns†

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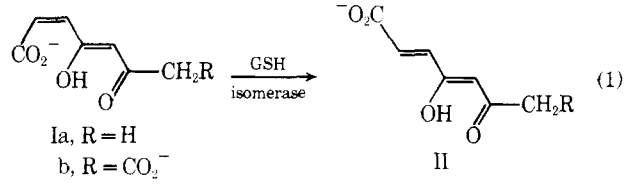
ABSTRACT: Maleylacetone *cis-trans*-isomerase isolated from *Vibrio 01* binds glutathione strongly; $K_m = 1.4 \times 10^{-4}$ M. Oxidized glutathione and *S*-methylglutathione are competitive inhibitors, $K_I = 9.4 \times 10^{-4}$ and 1.2×10^{-3} M, respectively. Based on these interactions, three different glutathione-bound agarose affinity adsorbents were synthesized and tested. Affinity chromatography of the isomerase with one of these affords 70- to 100-fold purifications. In separate syntheses, portions of the affinity arm were prepared and examined as to their inhibitory properties in the enzyme-catalyzed reaction. The fragment, containing glutathione bound through its sulfur to the carbon chain, is a powerful competitive inhibitor for glutathione ($K_I = 6 \times 10^{-5}$ M). The results described suggest that the isomerase binds glutathione through the backbone of the tripeptide and that the thiol group is required for activity. The initial velocity patterns of the enzyme-catalyzed reaction resulting from simultaneous variation of glutathione and maleylacetone concentrations were examined in the absence and presence of inhibitors resembling glutathione. The observed kinetic patterns suggest an ordered sequence of binding: maleylacetone first followed by glutathione.

Although glutathione appears to be present in many organs (Jocelyn, 1972) to maintain proteins in what has been termed a euphoric state, i.e., a reduced state (Racker, 1954), there is a growing list of enzymes which specifically require GSH[1] for function. In a previous report from this laboratory, the isolation and purification of an enzyme from *Vibrio 01* which catalyzes cis–trans isomerization about the carbon–carbon double bonds of maleylacetone and maleylacetoacetate (eq 1) (Seltzer, 1973a) was described. Catalysis is dependent on the presence of GSH. The function of GSH in this reaction does not appear to be connected with its capacity as a reducing agent since sodium borohydride and other thiols such as mercaptoethanol, mercaptoethylamine, dithiothreitol, and cysteine were reported not to be able to replace GSH in the enzymatic reaction. Maleylacetoacetate *cis-trans*-isomerase from mammalian liver and maleylpyruvate *cis-trans*-isomerase from *Pseudomonas ovalis* also appear to require GSH for activity (Edwards and Knox, 1956; Lack, 1959, 1961).



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In this report experiments are described which show that GSH binds to maleylacetone *cis-trans*-isomerase. Two analogues of GSH, GSMe and GSSG, are shown to be competitive inhibitors for the coenzyme. A description of the utilization of this binding for enzyme purification, which includes the synthesis and examination of three different glutathione-bound agarose adsorbents, is given. Moreover, the synthesis of portions of the adsorbent arms and a study of their binding properties to the isomerase are described.

Experimental Section

^{1}H NMR spectra were obtained with a JEOL JNM-MH-100 MHz instrument, while ^{13}C NMR spectra were obtained on a Varian CFT-20.

Materials. GSH, GSMe, GSSG were commercial products of Sigma Chemical Co. GSSG was purified by the method of Furano (1971) on a Dowex 1-X2 column using a water–2 N acetic acid linear gradient. GSH and GSMe were used without further purification. Bovine serum albumin was a product of Armour. Cyanogen bromide and mercaptoethanol were obtained from Eastman Organic Chemical Co. *N,N'*-Dicyclohexylcarbodiimide, maleic anhydride, and 1,6-diaminohexane were obtained from the Aldrich Chemical Co. DNTB was a Calbiochem product. Maleylacetone was prepared as previously described (Fowler and Seltzer, 1970). *n*-Hexylamine was Baker grade from the Baker Chemical Co. *Vibrio 01* were grown at the New England Enzyme Center and the maleylacetone *cis-trans*-isomerase obtained from it was purified as described previously (Seltzer, 1973a), except where noted differently.

N-(Aminohexyl)maleamic Acid. 1,6-Diaminohexane (9.5 g) in 200 ml of dioxane was added rapidly with mixing to a solution of 10 g of maleic anhydride in 100 ml of dioxane cooled in ice. After 20 h at room temperature, the dioxane was decanted and the residue dried in a stream of nitrogen. A small amount of the solid, dissolved in warm D_2O, exhibited the following NMR peaks: δ 5.86 and 6.23 (vinyl quartet, $J = 12.3$ Hz, 2 H), 2.99 (multiplet, 4 H), 1.39 (broad, 8 H) with internal dioxane at δ 3.65.

N-Hexylmaleamic Acid. N-Hexylamine (9.7 g) was added dropwise with stirring to a solution of maleic anhydride (9.4 g) in dioxane (100 ml) at 4 °C. After addition was completed, stirring was continued for about 3 h at room temperature. The

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[1] Abbreviations used: GSH, glutathione; GSMe, *S*-methylglutathione; GSSG, oxidized glutathione; DNTB, 5,5'-dithiobis(2-nitrobenzoic acid); CNBr, cyanogen bromide; NMR, nuclear magnetic resonance.

mixture which was filtered after overnight refrigeration was recrystallized from dioxane and dried in vacuo: mp 75.9–77.5 °C [reported: 78 (Mehta et al., 1960); 76 °C (Liwschitz et al., 1956)]; ^{1}H NMR ($CDCl_3$) δ 0.91 (triplet, 3 H), 1.36 and 1.64 (poorly resolved multiplet, 9 H), 3.46 (overlapping triplets, $J \sim 7$ Hz, 2 H), 6.41 and 6.75 (AB quartet, $J = 13.5$ Hz, 2 H), and 8.68 (broad, 1 H, carboxyl); ^{13}C NMR (proton decoupled in $CDCl_3$) 166.98 (50), 166.41 (57), 135.34 (95), 132.91 (64), 40.72 (83), 31.44 (78), 28.61 (84), 26.64 (67), 22.56 (68), 13.98 (43), 0.00 (97) (Me_4Si).

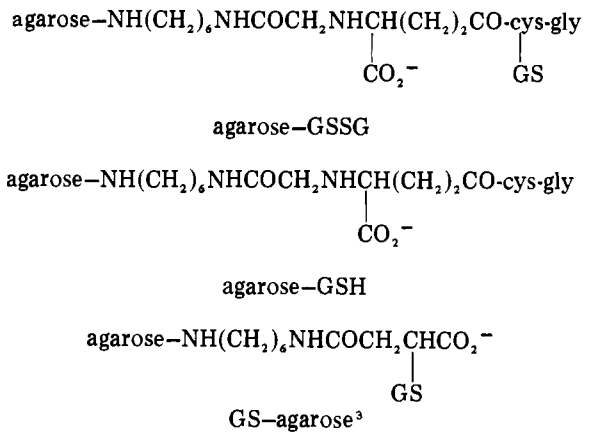
α- and β-S-Glutathiyl-N-hexylmaleamate. Conjugate addition of GSH to *N*-hexylmaleamate was carried out in D_2O in order to monitor it by NMR. GSH (5.17 mmol) was added to a solution of 4.94 mmol of *N*-hexylmaleamic acid in 16.6 ml of D_2O containing a fourfold excess of sodium bicarbonate. After 6 days 90% of the vinyl resonance disappeared at which point the mixture was worked up. The desired compound was twice chromatographed on a column of Bio-Rad AG11A8 resin eluted with water, yield 0.73 g. Anal. Calcd for $C_{20}H_{32}O_9N_4SNa_2 \cdot 2H_2O$: C, 40.95; H, 6.19; S, 5.46. Found: C, 40.60; H, 6.25; S, 5.66. The residual free sulfhydryl of the product was determined with DNTB and found to be <0.025 mol % of the total product present.

Kinetic Studies. The isomerase, used for inhibition studies with GSSG and GSMe, was purified as previously described (Seltzer, 1973a). For studies with *N*-hexylmaleamate and α- and β-*S*-glutathiyl-*N*-hexylmaleamate, the isomerase, after the calcium phosphate gel chromatography step, was subjected to affinity chromatography on the GS-agarose adsorbent as described in the Results. Initial velocity measurements were carried out at pH 7.4 in 0.01 M phosphate buffer, except for studies with *N*-hexylmaleamate which were with 0.1 M phosphate. Catalyzed reactions were quenched with sufficient sodium hydroxide to bring the assay solution rapidly to pH 13. Absorbancies at 298.5, 322, and 345 nm were quickly measured to determine the extent of isomerization as previously described (Seltzer, 1973a). Reaction times were chosen to ensure that less than 20% of the initial substrate was isomerized.

Results

Inhibition Studies with GSSG and GSMe. Preliminary rate measurements indicated that the GSSG obtained commercially contained some GSH since it caused the enzyme to catalyze the isomerization in the absence of added GSH. GSSG, chromatographically purified on Dowex 1-X2, however, could not stimulate isomerization when added to a solution containing enzyme and substrate. GSMe, on the other hand, appeared to have no underivatized GSH present since it could not stimulate reaction in the absence of added GSH. Inhibition studies with GSSG and GSMe were carried out with constant concentrations of enzyme and maleylacetone while the concentration of GSH was varied in five steps from 2.5×10^{-5} to 4.3×10^{-4} M and the GSH analogue inhibitor concentration was varied in five steps from zero to 1.5×10^{-3} M. Hence 25 different combinations (5 × 5 arrays) of GSH and GSH analogue concentrations were examined for each inhibition study. Initial velocities were least-squares fitted to $v = VA/(K + A)$ (Cleland, 1967), where A is the GSH concentration at constant inhibitor and maleylacetone concentrations. The action of both analogues results in competitive inhibition. (V_{max} so obtained for each inhibitor concentration varies from the average V_{max} by about ±10% which is the same as the average standard deviation in the determination of each individual V_{max}.) The slopes (i.e., the K/V_{max} terms of a double-reciprocal plot) also have an average ±10% deviation associated with them. A replot of the slope terms vs. inhibitor concentration indicates that both GSSG and GSMe are linear competitive inhibitors for GSH. Least-squares solution for the intercepts and slopes of the replots yield K_I(GSSG) = 9.4×10^{-4} M and K_I(GSMe) = 1.2×10^{-3} M.[2]

GSH-Affinity Adsorbents. Three different derivatized agarose adsorbents were prepared in which glutathione was bound differently in each but always resided at the end of a chain which in turn was covalently bound to agarose. The condensed structures of the three adsorbents are shown below.



Agarose–GSSG. In early attempts GSSG and GSH were bound through their free amino groups. Agarose was altered in its standard way for attachment of a peptide through an amino group (Cuatrecasas and Anfinsen, 1971). Cyanogen bromide activated agarose was treated with 1,6-diaminohexane at pH 10.5 and the product subsequently derivatized with succinimidyl *N*-(bromoacetate). Addition of GSSG at pH 9 afforded displacement of the α-bromine by one of the amino groups of the hexapeptide, after which, remaining active groups were scavenged by 0.6 M aminoethanol.

Agarose–GSH was prepared by thiol–disulfide interchange of dithiothreitol (8 mM, pH 7.4) with agarose–GSSG. After thorough washing, the product was estimated (Ellman, 1958) to have 0.02 μmol of GSH/ml of agarose.

In the third adsorbent, *GS–agarose*, glutathione was bound to the added chain through its sulfur. The synthesized *N*-(aminohexyl)maleamate was added to 103 ml of packed cyanogen bromide activated agarose at pH 10.2. The resulting derivatized agarose, having a conjugated carbon–carbon double bond, was treated with GSH (80 mM) at neutral pH to facilitate attack by SH rather than by its amino group. The addition of thiols to conjugated α,β-unsaturated systems is known to be rapid (Seltzer, 1973a). The sulfur content of a thoroughly washed and dried sample, as determined by x-ray fluorescence,[4] was 0.97%, while that for underivatized agarose was 0.17%.

Affinity Studies with Glutathione–Agarose Adsorbents. Results of attempted enzyme purification on agarose–GSH were erratic. After crude enzyme was applied to the column,

[2] The authors have been urged by the editors to delete all linear plots from the manuscript and to substitute their slopes and intercepts. Reproductions of the actual plots are available from the authors upon request.

[3] Thiol addition is suggested to occur mainly at the α carbon rather than at the β carbon because of the relative stabilities of the possible intermediate carbanions that would result.

[4] We thank Ms. E. Norton and Dr. R. Stoenner for the x-ray fluorescence analysis.

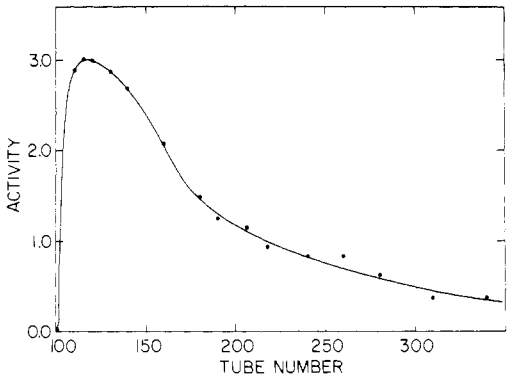


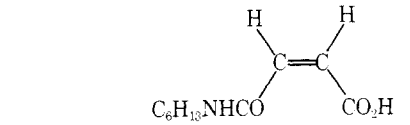
FIGURE 1: Typical elution profile of maleylacetone *cis-trans*-isomerase from a GS-agarose column. Six-milliliter fractions were collected. Activity is represented in arbitrary units. Enzyme (12 ml, 60.6 units, specific activity, 0.788 unit/mg), which had been partially purified up to and including the calcium phosphate gel chromatography step (Seltzer, 1973a), was applied to the GS-agarose column (1.5 × 50 cm). Elution of contaminating protein by 0.03 M phosphate, pH 7.4, was carried out until the A_{280} of the eluate was negligible. The isomerase was then eluted with 5 × 10^{-3} M GSH in 0.03 M phosphate buffer at a flow rate of 0.4 ml/min. Active fractions (tubes 100 to 340) were pooled and concentrated by ultrafiltration and then dialyzed to remove GSH. Recovered activity amounted to 54.1 units (specific activity, 72.8 units/mg, 92-fold purification) for a yield of 89%. Protein concentrations were determined by first hydrolyzing the eluted isomerase with 6 N HCl for 20 h at 110 °C and then determining the α-amino acid concentration with ninhydrin. Leucine was used as a standard. Disc gel electrophoresis of the enzyme isolated after affinity chromatography indicates that it is not homogeneous. Purification can be achieved by gel filtration on Sephadex G-75. Similar results were obtained with another batch of GS-agarose synthesized at a different time. To a column (1.5 × 25 cm) of adsorbent, 30.3 units (specific activity, 0.788 unit/mg) of maleylacetone *cis-trans*-isomerase was added. Recovered isomerase amounted to 28.9 units (95% recovery) with a specific activity of 66 units/mg, representing an 84-fold purification.

the column washed with 0.01 M phosphate, and the enzyme eluted with a GSH solution, only a twofold purification could be realized. Attempted reuse of the column was unsuccessful perhaps because of the thiol oxidation of the bound GSH.

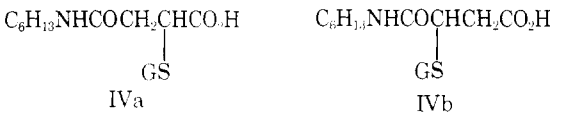
Similar studies were carried out with the agarose-GSSG adsorbent. Crude enzyme applied to this column could be purified 12-fold when it was eluted with GSH. Agarose-GSSG (60 ml) was packed in an 18-mm o.d. column. Crude enzyme (8 ml, ~1 mg/ml, specific activity, 0.1 μmol min^{-1} mg^{-1}) in 0.01 M phosphate buffer, pH 7.4, containing 0.1% mercaptoethanol was added to the column. The column was washed with (a) 60 ml of 0.01 M phosphate buffer, (b) 200 ml of the buffer containing 363 mg of GSSG, and (c) 200 ml of the buffer containing 308 mg of GSH. All active fractions (210 ml) were combined and concentrated yielding isomerase with a specific activity of 1.2. Use of the column a second time resulted in a smaller purification factor. Continued use resulted in loss of affinity probably because of thiol–disulfide interchange and subsequent oxidation of the bound GSH.

The GS-agarose proved to be a stable adsorbent with considerably greater affinity for the enzyme. In these studies the crude enzyme was applied to the column and separated from contaminating protein by washing the column with 0.03 M phosphate, pH 7.4. The isomerase (specific activity ~ 70 units/mg) is then eluted with 5 mM GSH in 0.03 M phosphate buffer (see Figure 1). The column has been reused several times with no apparent loss of affinity resulting in purifications of 70- to 100-fold. Analysis for sulfur in the GS-agarose adsorbent shows that about 250 μmol of GS is bonded/g of anhydrous GS-agarose. An adsorbent column in a Pasteur pipet prepared from 1.6 ml of the GS-agarose adsorbent (42 mg anhydrous weight) was overloaded with enzyme; 0.65 unit was extracted by the adsorbent. The enzyme was freed from the column with GSH in the usual way; 0.80 unit was eluted. Thus 1 μmol of agarose-bound GSH appears to hold about 3 × 10^{-5} μmol of enzyme (mol wt ~ 35 000).

Investigations Concerned with the Nature of Enzyme Binding to the GS-Agarose Adsorbent. Two substrate analogues, III and IV, were prepared in order to study the binding of the enzyme to various portions of the adsorbent arms that may be present in the matrix. Compound III was synthesized



III



IVa IVb

previously; the cis orientation at its double bond is verified by the coupling constant for its vinyl protons [J = 13.5 Hz (12.5 Hz in D_2O, pD 7)]. Reaction of GSH with III results in the loss of the β-CH_2 ^1H NMR resonance of the cysteine of GSH and the vinyl resonance of III. These are replaced by two broad resonances 0.1 and 0.2 ppm downfield from the original position of the cysteine β-CH_2 doublet. The fact that the new resonances are multiplets suggest that a mixture of IVa and IVb is present. No attempt was made to separate them because of their highly similar nature. The structure assigned to the product is further supported by elemental analysis and the fact that analysis with DNTB indicates no free sulfhydryl present.

The inhibition patterns of III and IV were investigated by holding one substrate concentration (GSH or maleylacetone) constant while allowing the other to vary. This was repeated for several inhibitor concentrations. Initial velocities of isomerization at constant concentration of inhibitor and one substrate were least-squares-fitted to $v = VA/(K + A)$ where A is the concentration of the variable substrate. The K/V ("slope") and $1/V$ ("intercept") values obtained were plotted vs. the concentration of inhibitor. The slopes and intercepts obtained from the least-squares solutions of these summary plots are given in Table I.

In the absence of the actual plots,[2] it is difficult for the reader to decide whether the slope of a summary plot is or is not sensitive to variation in the concentration of inhibitor. For this reason values of a "5% slope", in the dimensions of the plot, are included in Table I. These values are obtained by taking 5% of the value of the intercept divided by the inhibitor concentration range studied. As can be seen from the table, both slopes and intercepts are sensitive to the concentration of inhibitor III when either GSH or maleylacetone is the variable substrate. Therefore III is a *noncompetitive* inhibitor for both maleylacetone and GSH. Inhibition is somewhat weak requiring high concentrations (~10^{-2} M) in order to see an effect on the rate. Inhibition by IV, however, is very effective in the 10^{-4} M range. It can be seen that when GSH is the variable substrate the slopes of double-reciprocal plots are sensitive to the concentration of IV but the intercepts of these plots are relatively insensitive. This indicates that IV is a *competitive* inhibitor for GSH (K_I = 6 × 10^{-5} M). When maleylacetone is the variable substrate the reverse is observed; intercepts are sensitive but slopes are insensitive. This shows that IV is an *uncompetitive* inhibitor for maleylacetone. The plots suggest

TABLE I: Inhibition Characteristics of Maleylacetone *cis-trans*-Isomerase by Compounds III and IV.

Inhibitor[a]	Variable Substrate	Replot of [b]	Least-Squares Values: Slope	Least-Squares Values: Intercept	5% Slope[c]	Type[d]
III	GSH	S	3.3×10^{-4}	1.5×10^{-5}	$\pm 4.2 \times 10^{-5}$	NC
III	GSH	I	1.8	2.4×10^{-2}	$\pm 6.5 \times 10^{-2}$	
III	Maleylacetone	S	2.5	5.5×10^{-2}	$\pm 1.6 \times 10^{-1}$	NC
III	Maleylacetone	I	2.3×10^{3}	1.2×10^{1}	$\pm 3.6 \times 10^{1}$	
IV	GSH	S	3.8×10^{-1}	2.3×10^{-5}	$\pm 2.7 \times 10^{-3}$	C
IV	GSH	I	-5.2×10^{2}	1.4	$\pm 1.6 \times 10^{2}$	
IV	Maleylacetone	S	2.2×10^{2}	1.3	$\pm 1.4 \times 10^{2}$	UC
IV	Maleylacetone	I	5.1×10^{6}	1.4×10^{3}	$\pm 1.6 \times 10^{5}$	

[a] Inhibitor concentrations for III varied between 0 and 18 mM, while for IV it varied between 0 and 0.44 mM. [b] S = slopes, I = intercepts. [c] The 5% slope = 0.05 × intercept value/inhibitor concentration range studied. [d] NC = noncompetitive; C = competitive; UC = uncompetitive. The assignment is based on whether the slope or intercept is relatively constant over the range of inhibitor concentration studied.

that both inhibitors are linear in their behavior over the concentration range studied.

It was of interest to determine the kinetic form of the enzyme-catalyzed reaction in the absence of inhibitors. At each of four maleylacetone concentrations, initial rates were determined for six different concentrations of GSH. Rates for different concentrations of GSH, but for the same concentration of maleylacetone, were least squares-fitted (Cleland, 1967) to $v = VA/(K + A)$ where A is the GSH concentration. The slope (K/V terms) and intercept ($1/V$ terms) quantities obtained at each maleylacetone concentration were then plotted against the reciprocals of the corresponding maleylacetone concentrations used. The slopes and intercepts of the linear least-squares computed lines are as follows: (K/V)'s vs. [maleylacetone]$^{-1}$, slope = 2.9×10^{-4}, intercept = 1.7×10^{-1}; for $(1/V)$'s vs. [maleylacetone]$^{-1}$, slope = 8.6×10^{-1}, intercept = 1.2×10^{3}. The nonzero intercept of these lines and the good fit of the $1/v$ vs. $1/[\text{GSH}]$ plots suggest that the kinetics follow eq 2

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \qquad (2)$$

(Cleland, 1970) where A and B are the GSH and maleylacetone concentrations, respectively. The values derived from the least-squares solutions are $K_b = 7.5 \times 10^{-4}$ M, $K_{ia} = 3.4 \times 10^{-4}$ M, and $K_a = 1.4 \times 10^{-4}$ M.

Discussion

It is apparent from these studies that GSH is required for maleylacetone *cis-trans*-isomerase activity. The current results show that a limit in enzymatic activity obtains upon increasing the concentration of GSH which indicates that a site for GSH-binding exists on the isomerase. K_m(GSH) was found to equal 0.14 mM.

In a previously postulated mechanism the sulfhydryl group was suggested to act as a nucleophile toward the conjugated carbon–carbon double bond (Seltzer, 1973b) which follows from earlier studies on model systems (Seltzer, 1959; Stevens and Seltzer, 1968). Similar hypotheses have been advanced by Lack (1959, 1961) concerning the enzyme-catalyzed isomerization of maleylpyruvate. Neither GSMe nor GSSG can replace GSH in the maleylacetone enzymatic reaction, a fact which is consistent with the notion that the sulfhydryl group of the coenzyme is necessary for reaction. Both analogues are competitive inhibitors which bind to the enzyme, by a factor between five to ten times, less tightly than GSH. Although the sulfhydryl is required for reaction it appears not to be required for binding to the enzyme. These data suggest that the enzyme is bound through its backbone structure while the sulfhydryl points away from the protein surface. This conclusion is further supported by studies with affinity adsorbents.

To our knowledge only one previous study concerned with a glutathione-bound agarose adsorbent is recorded and this was reported while our work was in progress (Brocklehurst et al., 1974). In that system, anchored glutathione is treated with 2,2′-dipyridyl disulfide to form a bound mixed disulfide. Utilization of this adsorbent rests on the ability of a thiol enzyme such as papain to enter into thiol–disulfide interchange with the adsorbent resulting in covalent attachment and immobilization of the enzyme. The function of glutathione in the reported adsorbent, which is rather nonspecific, is to provide a sizable spacer between the amino group, needed for attachment, and the thiol, required for disulfide formation. Amine and thiol groups separated instead by a hydrocarbon chain would probably do as well in that system (Brocklehurst et al., 1974). Since maleylacetone *cis-trans*-isomerase requires GSH as a coenzyme, it was anticipated that the interaction between anchored GSH and isomerase would be highly specific and this is borne out by the studies described here.

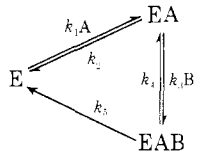
Three different glutathione adsorbents were synthesized. The enzyme binds to each of these in the *absence* of maleylacetone. Relatively crude enzyme, applied to each of the affinity adsorbents, could be substantially purified by preliminary elution of weakly held proteins with buffer and final elution of the isomerase with GSH. The purification factor achieved with each of the adsorbents was in the order: GS–agarose ≫ agarose–GSSG > agarose–GSH. Affinity chromatography of partially purified isomerase on GS–agarose can result in a purification factor of 70- to 100-fold and a recovery of about 90% of the activity. The adsorbent seems to be more specific for isomerase when the backbone of a γ-Glu-Cys-Gly moiety is free. It is apparent that here too the sulfhydryl is unnecessary for binding to the enzyme.

That the attachment of isomerase to the adsorbent results from the binding of the enzyme to the backbone of the an-

chored glutathione is shown by inhibition studies with compounds III and IV. III and IV were synthesized to mimic parts of the adsorbent arms that may be bonded to the agarose. Both these compounds were found to inhibit the enzymatic reaction. The results are summarized in Table I.

As expected, IV binds strongly to enzyme ($K_I = 6 \times 10^{-5}$ M) and is a *competitive* inhibitor for GSH. This is in agreement with the observation that the isomerase can be discharged from the affinity adsorbent when the eluting buffer contains free GSH. It is interesting that IV is an *uncompetitive* inhibitor for maleylacetone. It might be thought that IV, being the adduct of GSH and a residue resembling in part maleylacetone, might have features which would resemble a possible addition complex of maleylacetone and GSH. In that case IV might have been expected to be a competitive inhibitor against both maleylacetone and GSH. This is not the case; the least-squares solution to a replot of the slope, in runs where maleylacetone is the variable substrate, vs. the concentration of IV, is essentially horizontal (actually slightly negative) indicating that the enzyme only binds to the anchored GSH residue.

Further examination of the inhibition patterns leads to interesting implications regarding the sequence of binding of the two substrates, maleylacetone and GSH, which in turn may help in understanding the mechanism of reaction. But first we must discuss the kinetic pattern that obtains in the absence of inhibitors. As shown the kinetics follow eq 2. Mechanisms which yield kinetics of the form shown in eq 2 are the sequential ordered BiBi (eq 3) and the rapid equilibrium random BiBi (eq 4).



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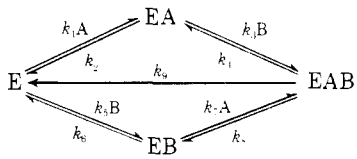

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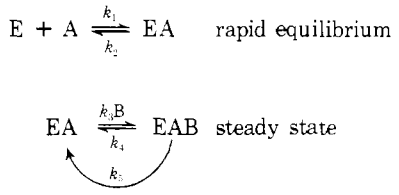
A special case of these which also fits is the Theorell–Chance mechanism where the steady-state concentration of EAB approaches zero. The present results rule out a ping-pong mechanism and those shown in eq 5 and 6



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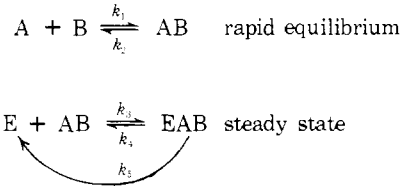
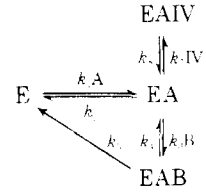


(6)

since they would not fit eq 2 either. Were GSH to bind at a site distant from the maleylacetone site and to act as an activator in the reaction with no direct specific interaction with maleylacetone, a mechanism of the type shown in eq 5 (A = GSH) might be expected. This type of mechanism, were it operating, could be demonstrated by initial rate studies. If GSH were the variable substrate, accordingly a replot of the "slopes" vs. the reciprocal of the maleylacetone concentrations would be expected to give a straight line that would go through the origin. Moreover, a replot of the "intercepts" vs. the reciprocal of the maleylacetone concentration would be expected to give a horizontal line. As can be seen from the data, this is not the case. The observed kinetics suggest that both A and B or their product counterparts are discharged from the enzyme surface at each turnover. This could mean that they leave separately or covalently bonded as in an adduct intermediate. If GSH were an activator it might be expected to remain fixed at its site for many turnovers.

If GSH and maleylacetone were to combine to form a complex (eg., a thiohemiketal) in a rapid equilibrium which then combines with enzyme, then a mechanism as shown in eq 6 would be followed. No such complex can be detected by NMR, however, and therefore an upper limit to its concentration is about 1% of the maleylacetone present. Under the condition where the fraction of substrate in the form of a complex (i.e., AB) is small and the mechanism follows the scheme shown in eq 6 then a replot of the "intercepts" vs. the reciprocal of the maleylacetone concentration would yield a straight horizontal line. This is clearly not the case and we therefore reject this mechanism too. Thus while the results rule out some mechanisms they do not differentiate between an ordered sequential and a rapid equilibrium random BiBi mechanism. Further results discussed below suggest that an ordered sequential mechanism operates.

Compound IV, an analogue of GSH, binds more tightly to the isomerase than does the coenzyme. As pointed out it is a competitive inhibitor for GSH but an uncompetitive inhibitor for maleylacetone. This suggests that the predominant binding sequence is that shown in eq 7



(7)

where maleylacetone (A) binds first followed by GSH (B). Competitive inhibition is satisfied by recognizing that either GSH or IV can combine with the maleylacetone-enzyme complex (EA). At constant concentrations of IV and B the ratio of inhibited form (EAIV) to active form (EAB) remains fixed. Increasing the concentration of A increases the rate because the ratio of EA to E increases. Inhibition by IV, however, cannot be reversed by increasing A and thus IV will be expected to be an uncompetitive inhibitor against maleylacetone. Were GSH to bind to enzyme before maleylacetone, then IV would be expected to be a competitive inhibitor against GSH but a *noncompetitive* inhibitor against maleylacetone.

The mechanistic implications of the inhibition patterns exhibited by III appear less significant. Since III has features resembling maleylacetone, it might have been expected to be a competitive inhibitor for maleylacetone. It is a weak noncompetitive inhibitor for both maleylacetone ($K_I = 2 \times 10^{-2}$ M) and GSH ($K_I = 4 \times 10^{-2}$ M). Consequently the high concentrations of III that are required for inhibition may sufficiently perturb the system making it difficult to interpret the results. We prefer not to speculate about the inhibition patterns for III.

As discussed above the pattern exhibited by IV leads us to postulate that the predominant path is for maleylacetone to

bind to enzyme before GSH does. It might appear that the observations from affinity chromatography suggest a different order of binding since the enzyme attaches to adsorbent, having active arms resembling IV, in the absence of maleylacetone. This is not really a contradiction. Free enzyme might be able to bind GSH in the absence of maleylacetone but may result in a nonproductive complex. There are a few cases of ordered sequential mechanisms where high concentrations of the second substrate appear to inhibit the reaction because binding of the second substrate first leads to an inactive species incapable of further reaction unless it first dissociates (Dalziel, 1957).

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Conformational Changes in Subfractions of Calf Thymus Histone H1†

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ABSTRACT: This paper presents the first study of conformational changes in the subfractions of calf thymus H1. H1 was fractionated by the method of Kincade and Cole (Kincade, J. M., and Cole, R. D. (1966), *J. Biol. Chem. 241,* 5790) using a very shallow Gdn-HCl gradient. A possible new H1 subfraction, about 5–8% of the H1, has been found and characterized by amino acid analysis and electrophoresis. The effects of salt concentration and pH on the conformation of each of the four major subfractions have been studied by measuring the fluorescence anisotropy of the tyrosine emission and the circular dichroism (CD) of the peptide bond. Upon the addition of salt to aqueous solutions at neutral pH, all four subfractions show an instantaneous change in fluorescence anisotropy, fluorescence intensity, tyrosine absorbance, and CD. The folding associated with this instantaneous change is highly cooperative, and involves the region of the molecule containing the lone tyrosine, which becomes buried in the folded form. The folding of subfraction 3a is more sensitive to salt than the other major subfractions. Upon folding, approximately 13% of the residues of subfractions 1b and 2 form α and β structure; 3a and 3b have approximately 16% of the residues in α and β structures. There is no evidence for interactions between the subfractions. In salt-free solutions, each of the four major subfractions shows very little change in conformation in going from low to neutral pH, but each shows a very sharp transition near pH 9. This transition gives rise to a marked increase in fluorescence anisotropy and fluorescence intensity, and involves the formation of both α and β structure in a manner similar to that of the salt-induced state.

Histones have been known since 1884 (Kossel, 1884), but it was only the discovery of the subunit structure of chromatin (Hewish and Burgoyne, 1973; Woodcock, 1973; Olins and Olins, 1973, 1974; Sahasrabuddhe and Van Holde, 1974; Kornberg, 1974; Noll, 1974) that established at least some of the functions of histones H2a, H2b, H3, and H4. Compared to our knowledge of the non-H1, or inner histones,[1] our understanding of H1 is meager indeed. It is known that H1 is heterogeneous (Kincade and Cole, 1966a,b; Bustin and Cole, 1968, 1969; Kincade, 1969; Panyim and Chalkley, 1969a; Fambrough and Bonner, 1969; Langan et al., 1971; Seale and Aronson, 1973; Stout and Phillips, 1973; Sherod et al., 1974; Ruderman et al., 1974; Ruderman and Gross, 1974; Gurley et al., 1975; Spiker, 1976), in contrast to the evolutionary conservation of the inner histones, especially H3 and H4 (DeLange et al., 1969; DeLange and Smith, 1971; Panyim et al., 1971). Evidence has been presented that H1 is, in some fashion, involved in chromatin condensation and mitosis (Littau et al., 1965; Mirsky et al., 1968; Bradbury et al., 1973a), with the phosphorylation of H1 perhaps serving as a trigger for mitosis (Lake and Salzman, 1972; Lake, 1973;

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[1] In this paper, histones H2a, H2b, H3, and H4 will be called the *inner histones* (Isenberg, 1976), since they are inside of the nucleosomes.