Kinetic Analysis of the Mechanism of Insulin Degradation by Glutathione–Insulin Transhydrogenase (Thiol:Protein-Disulfide Oxidoreductase)†

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ABSTRACT: Kinetic studies have been made with glutathione-insulin transhydrogenase, an enzyme which degrades insulin by promoting cleavage of its disulfide bonds via sulfhydryl-disulfide interchange. The degradation of ^{125}I-labeled insulin by enzyme purified from beef pancreas was studied with various thiol-containing compounds as cosubstrates. The apparent K_m for insulin was found to be a function of the type and concentration of thiol; values obtained were in the range from 1 to 40 μM. Lineweaver-Burk plots for insulin as varied substrate were linear, whereas those for the thiol substrates were nonlinear: the plots for low molecular weight monothiols (GSH and mercaptoethanol) were parabolic; those for low molecular weight dithiols (dithiothreitol, dihydrolipoic acid, and 2,3-dimercaptopropanol) were apparently linear modified by substrate inhibition; and the plots for protein polythiols (reduced insulin A and B chains and reduced ribonuclease) were parabolic with superposed substrate inhibition. The nonparallel nature of the reciprocal plots for all substrates shows that the enzyme does not follow a ping-pong mechanism. Product inhibition studies were performed with GSH as thiol substrate. Oxidized glutathione was found to be a linear competitive inhibitor vs. both GSH and insulin. The S-sulfonated derivative of insulin A chain was also linearly competitive vs. both substrates. Inhibition by S-sulfonated B chain was competitive vs. insulin; the data eliminated the possibility that this derivative was uncompetitive vs. GSH. Experiments with the cysteic acid derivatives of insulin A and B chains similarly excluded the possibility that these were uncompetitive vs. either substrate. These inhibition studies indicate that the enzyme probably follows a random mechanism.

The reductive cleavage of the disulfide bonds of insulin by an extract of rat liver was first reported by Narahara and Williams (1959). In the same year Tomizawa and Halsey (1959) reported the isolation of a homogeneous preparation of insulin-degrading enzyme from beef liver; this enzyme was later shown to degrade insulin by promoting the cleavage of its disulfide bonds (Tomizawa, 1962; Katzen and Stetten, 1962; Tomizawa and Varandani, 1965; Varandani and Tomizawa, 1966; Varandani and Nafz, 1969; 1970a; Varandani, 1972) via sulfhydryl-disulfide interchange (Varandani, 1966a; Katzen and Tietze, 1966) (Scheme I).

Scheme I

$$\text{insulin} + \text{GSH} \rightleftharpoons \text{GSSG} + \text{A chain} + \text{B Chain}$$

Since that time, this enzyme, glutathione-insulin transhydrogenase[1] has been isolated from beef (Tomizawa and Halsey, 1959; Katzen and Stetten, 1962), rat (Varandani, 1972), and human (Tomizawa and Varandani, 1965) liver, human kidney (Varandani and Nafz, 1969), beef pancreas (Varandani and Tomizawa, 1966), and beef heart (Chandler and Varandani, 1972). A systematic study of the distribution of this enzyme in the rat confirmed its ubiquitous occurrence in all the 13 tissues examined (Chandler and Varandani, 1972). The action of GIT[2] has been found to be the first and rate-controlling step in the disposition of insulin (Varandani et al., 1972; Varandani, 1973b,d), and hepatic GIT has been demonstrated to be under feedback control by insulin in the rat (Varandani et al., 1971; Varandani, 1974a; Thomas and Wakefield, 1973). Subcellular studies with rat liver have shown that the majority of the enzyme occurs in the microsomal fraction with small amounts also present in the soluble (Ansorge et al., 1973b; Varandani, 1973c) and plasma membrane (Varandani, 1973a) fractions.

While insulin appears to be the most active naturally occurring disulfide substrate (Varandani et al., 1975), GIT also possesses some activity with other disulfide-containing proteins (Katzen and Stetten, 1962; Katzen and Tietze, 1966; Varandani, 1973c; Varandani and Nafz, 1970b). In fact evidence has been presented (Tomizawa and Varandani, 1965; Katzen and Tietze, 1966; Varandani, 1973c; Ansorge et al., 1973a; Varandani, 1974b) that GIT has many similar properties to those of the "ribonuclease reactivating

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[1] The Enzyme Commission accepted name for this enzyme is thiol:protein-disulfide oxidoreductase (EC 1.8.4.2). It has recently been suggested (Askelof et al., 1974) that the enzyme(s) that catalyzes the sulfhydryl-disulfide interchange reaction should be considered a "thioltransferase" rather than a "transhydrogenase"; the designation glutathione-insulin transhydrogenase has been retained for consistency with previous publications to avoid further nomenclature confusion.

[2] Abbreviations used are: GIT, glutathione-insulin transhydrogenase; ASH, insulin A chain with all four cysteyl groups in the thiol form; BSH, insulin B chain with both cysteinyl groups in the thiol forms; $ASSO_3$ and $BSSO_3$, the A and B chains with S-sulfonated forms; ASO_3 and BSO_3, the A and B chains with the cysteine groups oxidized to cysteic acid; and RSH, ribonuclease with all eight cysteinyl groups in the thiol form.

enzyme" (Venetianer and Straub, 1963; Goldberger et al., 1963; Fuchs et al., 1967), and it is possible that these two preparations might be the same enzyme. In addition to thiols of low molecular weight such as reduced glutathione, mercaptoethanol, and cysteine (Varandani and Tomizawa, 1966), it has been recently found that GIT utilizes the thiol groups of some high molecular weight sulfhydryl-containing proteins such as alcohol dehydrogenase, glycerol-phosphate dehydrogenase, fructose 1,6-diphosphatase, reduced ribonuclease, and reduced A and B chains as thiol substrate (Chandler and Varandani, 1973b). Thus GIT is an unusual enzyme in that it promotes an interchange reaction between two proteins.

The few kinetic studies of GIT (Katzen and Stetten, 1962; Tomizawa and Varandani, 1965; Varandani and Tomizawa, 1966; Spolter and Vogel, 1968; Varandani, 1972; Chandler and Varandani, 1972; Ansorge et al., 1973b) have been concerned primarily with the determination of the qualitative properties of the enzyme, such as its apparent K_m values for insulin. Consequently, little of the information necessary for understanding the mechanism of action of this enzyme is available; the kinetics of this enzyme are of special interest since it deals with macromolecular substrates. The present study was undertaken to provide some of this information, and includes kinetic studies of insulin degradation with various thiol substrates as well as product inhibition studies.

Materials and Methods

GIT was purified from beef pancreas as previously described (Varandani and Tomizawa, 1966; Varandani and Nafz, 1970a). It was a single-component material as judged by starch block electrophoresis, by sedimentation in the ultracentrifuge, and by Sephadex G-100 chromatography; double-immunodiffusion of pancreatic GIT with rabbit antiserum to purified rat liver GIT gave a single band of precipitation. The molecular weight of this preparation was found to be 54,000 by sedimentation equilibrium studies and 58,000 by filtration on Sephadex G-100.

^{125}I-labeled insulin was prepared by the chloramine-T method (Varandani, 1967, 1968) and contained an average of 0.6 atom of iodine per molecule with 90% of the label attached to the A chain and 10% to the B chain. Labeled insulin was purified on Sephadex G-50 and by dialysis immediately before use (Chandler and Varandani, 1972); greater than 90% of the purified [^{125}I]insulin was bound by excess insulin-antibody. Reduced insulin A and B chains (ASH and BSH) and S-sulfonated derivatives ($ASSO_3$ and $BSSO_3$) were prepared as previously described (Varandani, 1966b). The cysteic acid derivatives of A and B chains (ASO_3 and BSO_3) were prepared by the oxidation of insulin with performic acid (Hirs, 1967). All insulin derivatives were purified by chromatography on Sephadex G-75 (Varandani, 1966b). Reduced ribonuclease (RSH) was prepared by the reduction of ribonuclease A with mercaptoethylamine in 0.1 M acetate–8 M urea at pH 4.5 and was freed of reagents by extensive dialysis against 0.5 M acetic acid.

Insulin degradation was measured (Chandler and Varandani, 1972, 1973b) by the conversion of ^{125}I-labeled insulin to a form soluble in 5% trichloroacetic acid (i.e., A chain (Tomizawa, 1962; Katzen and Stetten, 1962; Tomizawa and Varandani, 1965; Varandani and Tomizawa, 1966; Varandani, 1972)). The reaction mixture, consisting of a tracer amount of ^{125}I-labeled insulin, unlabeled insulin (0.037–27.0 μM), 4 mg of bovine serum albumin, and 2 μg of GIT in 0.9 ml of 0.15 M potassium phosphate–25 mM EDTA (pH 7.4) buffer, was equilibrated for 5 min at 37°. The thiol substrate was then added in 0.1 ml of the same buffer and after 2–10 min (see below) the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The precipitate formed was collected by centrifugation, and after the precipitate was washed with 2 ml of 5% trichloroacetic acid, the ^{125}I-content of the combined supernatants and of the precipitate (dissolved in 1.5 ml of 20% KOH) was determined by counting in a Packard autogamma spectrometer.

The rate of the reaction in this system has previously (Chandler and Varandani, 1973b) been shown to be linear with time and linear with respect to the amount of enzyme present, provided that the amount of insulin degraded is held to less than 10% of the total present. Therefore in the present study the time of incubation was chosen for each concentration of thiol such that this limit was not exceeded.

Control tubes with GIT omitted were run at each concentration of insulin and thiol compound. The nonenzymatic rate of insulin degradation varied directly with the concentration of each substrate and ranged from 0.15 to 1.13%/min. Thus, the percentage contribution of the nonenzymatic insulin degradation to the uncertainty in the measurements of the GIT-specific reaction was relatively constant over the entire range of the substrate concentration used. All rates reported have been corrected for nonenzymatic insulin degradation and so represent true enzyme-dependent rates; the ratio of enzymatic to nonenzymatic rates were in the range from 2 to 4.

The fitting of data to the various equations was performed by nonlinear regression using the Marquardt maximum neighborhood method (Marquardt, 1963; Daniel and Wood, 1971; Dammkoehler, 1966).

The measured values are the percentages of the insulin present degraded. The net degradation did not exceed 10% in any tube, and was usually between 2 and 7%. Thus the error in the measurement is reasonably constant over the entire range measured; the pooled standard deviation calculated from 50 duplicate measurements (Payne, 1972) was found to be 4.47% of the mean at the lower measured rates and 2.2% of the mean at the highest rates. The errors in the corresponding velocities (i.e., picomoles of insulin degraded per minute per microgram of enzyme) are not constant, however, since these values are computed by multiplying the net percent degraded by the concentration of insulin used. Therefore, to satisfy the necessity of constant variance (Wilkinson, 1961), all least-squares fits were made with the fraction of insulin degraded as the dependent variable. Thus, for the Michaelis–Menten equation

$$v = V_m S/(S + K_m) \quad (1)$$

data were actually fitted with the transformed equation

$$v/S = V_m/(S + K_m) \quad (2)$$

where v is the measured velocity, V_m is the apparent maximum velocity of the forward reaction, S is the concentration of insulin, K_m is the apparent K_m for insulin, and v/S is the observed fractional amount degraded of the insulin present. This transformation of the dependent variable of course has no effect on the fits to equations in which insulin is not a variable. All other equations given are shown in terms of v rather than v/S for consistency with the usual presentation, and the choice of symbols is according to that proposed by Cleland (1963a–c).

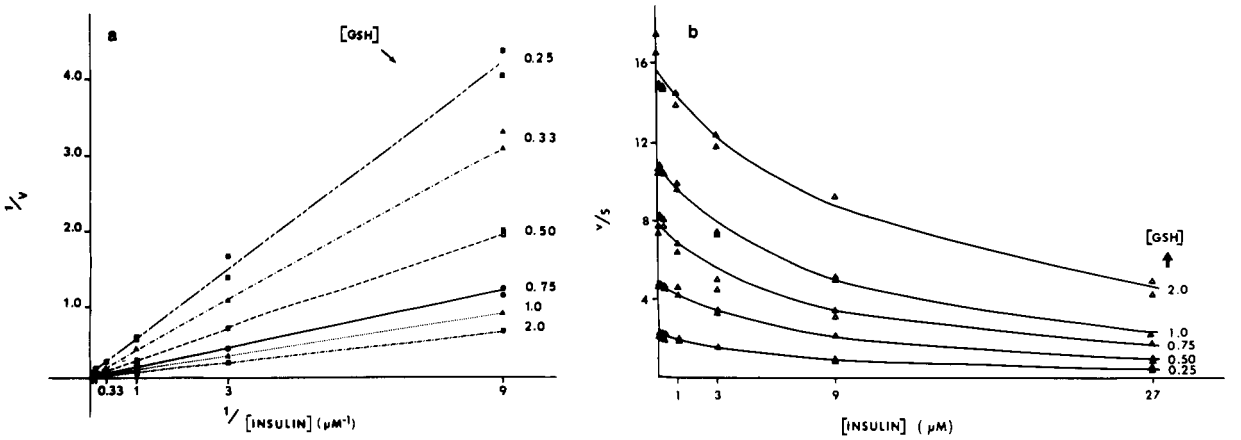


FIGURE 1: The initial velocity of insulin degradation catalyzed by GIT with insulin as variable substrate at fixed levels of GSH. The concentrations of GSH present (mM) were as indicated in the figure; v is expressed in picomoles of insulin degraded per minute per microgram of GIT. Experiments were performed as described in the text. Each symbol denotes an observed experimental point; the lines were derived by fits to the Michaelis-Menten equation (eq 1) to the data at each level of GSH as described in the text. In addition to the concentrations of insulin shown in the figures, all experiments were carried out using additional concentrations of insulin which are not illustrated due to problems of scale. Representative fits of eq 1 to the complete data sets are given in Table I. (a) Lineweaver-Burk reciprocal plot of the data; (b) v/S vs. S plot of the data shown in (a).

With the exception of the inhibition equations, all of the equations were fitted for each line separately to the appropriate data subset, e.g., the Michaelis-Menten equation was fitted to the data at each separate level of thiol substrate for the determination of V_m and K_m (insulin) values. The inhibition equations were fitted to the data subsets with one of the three variables (insulin, thiol, or inhibitor concentration) held constant.

Results

The basic kinetic experiment was carried out by varying the concentration of insulin from 0.037 to 27 μM at several fixed concentrations of GSH (0.167-2.0 mM). A double reciprocal plot of the data with insulin as variable substrate (Figure 1a) gave a series of nonparallel straight lines; representative values of the kinetic constants derived by fitting the Michaelis-Menten equation (eq 1) to the data are given in Table I. The value of V_m (the apparent maximum velocity) increased with the concentration of GSH present; the apparent K_m for insulin also appeared to increase, although this relation was obscured by the scatter in the data at low levels of GSH. The slope decreased with increasing levels of GSH; thus the possibility that the enzyme follows a ping-pong type mechanism is eliminated since for this mechanism the slopes should be constant (cf. Discussion).

A brief comment about the presentation of the data must be noted here. The data (in Figure 1a) and all succeeding plots are presented in reciprocal form, both for consistency with the usual presentation and to facilitate interpretation. However, it should be recalled that this format presents a distorted view in that it greatly exaggerates the residual errors at low as compared to high reaction velocities (Wilkinson, 1961). Conversely, a plot of v vs. [insulin] exaggerates the variance at high insulin concentrations, as discussed

Table I: Kinetic Constants for Insulin at Various Fixed Thiol Concentrations.[a]

Thiol Substrate	Thiol Concn (μM)	Apparent V_m (pmol min^{-1} μg^{-1})	Apparent K_m (insulin) (μM)	Slope (K_m/V_m)
Glutathione	250	14 ± 2	6.5 ± 0.8	0.451
	750	53 ± 10	6.7 ± 1.4	0.126
	2000	174 ± 20	11.1 ± 1.4	0.064
Mercaptoethanol	330	26 ± 5	11.0 ± 2.2	0.430
	1000	127 ± 16	19.4 ± 2.7	0.153
	3000	260 ± 38	21.1 ± 3.4	0.081
Reduced A chain	0.5	14 ± 5	5.4 ± 2.2	0.388
	2.0	102 ± 30	9.2 ± 2.9	0.090
	8.0	256 ± 150	24 ± 11	0.094
Reduced B chain	0.5	6 ± 3	2.9 ± 1.6	0.468
	2.0	47 ± 4	5.1 ± 0.5	0.109
	8.0	250 ± 42	21 ± 4	0.085
Reduced ribonuclease	0.11	7 ± 1	1.8 ± 0.4	0.257
	1.0	216 ± 23	13 ± 2	0.060
	9.0	330 ± 31	25 ± 3	0.074
Dihydrolipoic acid	4.1	9 ± 1	1.8 ± 0.2	0.204
	37	150 ± 10	7.2 ± 0.8	0.068
2,3-Dimercaptopropanol	37	27 ± 9	8.2 ± 3.2	0.308
	1000	260 ± 60	19 ± 5	0.073
Dithiothreitol	7.8	73 ± 4	10.0 ± 0.6	0.137
	31	159 ± 11	21.4 ± 1.6	0.134

[a] Initial velocity data were fitted with the Michaelis-Menten eq 1. The concentration of insulin was varied from 0.037 to 27 μM as described in text. The values given here are representative of the 6-10 concentrations of each thiol used. The error values given here and in succeeding tables are estimates obtained from the fitting procedure.

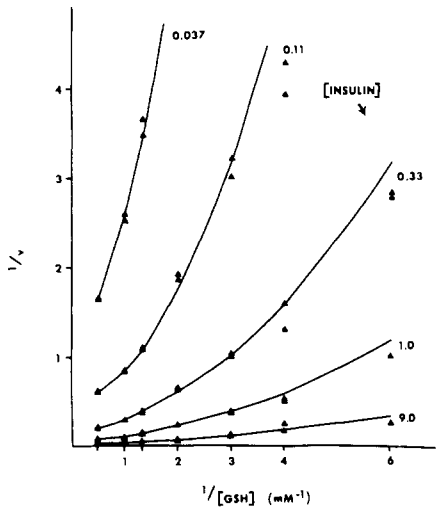


FIGURE 2: Insulin degradation by GIT with GSH as variable substrate at the indicated fixed concentrations of insulin (μM). Reciprocal plots of the initial velocity. v is expressed as picomoles of insulin degraded per minute per microgram of GIT. The symbols represent the observed points; the lines were determined by fits to the equation for a parabola (eq 3) to the data at each level of insulin. See Table II for representative fits of eq 3 to the complete data sets.

above (cf. Materials and Methods). Plots of the data as actually fitted, i.e., v/S vs. S (Figure 1b), demonstrate both the lack of bias in the fitted lines and the constancy of the variance in v/S over the entire range studied.

Double-reciprocal plots of the data with GSH as variable substrate (Figure 2) were nonlinear and appeared to be parabolas. Fitting the data with the equation (eq 3) for a parabola (in reciprocal form) indicated that this equation does

$$v = V_1/(1 + b_1/S + b_2/S^2) \quad (3)$$

in fact provide a good description of the data; the values of the fitted parameters are shown in Table II. The parabolic nature of these plots might indicate that either the reaction requires a minimum of two molecules of GSH per insulin molecule or that the reaction has alternate possible pathways. The parabolic nature of the data was confirmed by Hill plots of the data; the plots were linear and approximately parallel for all insulin concentrations. Direct fitting of the Hill equation (eq 4) to the data gave values of V_1

$$v = V_1/(1 + K/S^n) \quad (4)$$

similar to those obtained by the fits to eq 3 and values of n apparently independent of the insulin concentration with a mean for all insulin concentrations of 1.46 ± 0.15 (SD).

Mercaptoethanol. Experiments with varying levels of insulin (0.037–27.0 μM) and mercaptoethanol (0.2–3.0 mM) presented a picture essentially similar to that found with glutathione: reciprocal plots ($1/v$ vs. $1/S$) of the data with insulin as variable substrate were linear, while reciprocal plots with mercaptoethanol as variable substrate appeared parabolic. Fitting the Michaelis–Menten equation (eq 1) to the data at the various levels of mercaptoethanol gave the apparent V_m, K_m, and slope values shown in Table I. Hill plots of the data at fixed levels of insulin were linear, and fitting the Hill equation (eq 4) gave values of n apparently constant over all insulin concentrations with mean ± standard deviation of 1.25 ± 0.15. Consequently, a parabolic equation such as eq 3 is the simplest possible description of the data. Fits of eq 3 to the data gave the parameter values shown in Table II.

Thiol Proteins. The thiol proteins reduced insulin A chain (four sulfhydryl groups per molecule), reduced insulin B chain (two sulfhydryl groups per molecule), and reduced ribonuclease (eight sulfhydryl groups per molecule) all gave qualitatively similar results. Reciprocal plots with

Table II: Kinetic Constants for Thiols at Various Fixed Levels of Insulin.[a]

Thiol Substrate	Insulin Concn (μM)	Equation	Apparent V_1 (pmol min^{-1} μg^{-1})	b_1	b_2	K_I	Apparent K_m (thiol)
Glutathione	0.037	3	1.0 ± 0.1	1.10 ± 0.36	0.65 ± 0.07		1.52
(mM)	1.0	3	23 ± 3	0.87 ± 0.39	0.56 ± 0.08		1.30
	27	3	226 ± 184	1.0 ± 3.0	0.86 ± 0.46		1.56
Mercaptoethanol	0.037	3	0.7 ± 0.1	19 ± 1	0.18 ± 0.14		19.0
(mM)	1.0	3	18 ± 1	1.6 ± 0.3	0.18 ± 0.08		1.7
	27	3	281 ± 68	2.2 ± 1.3	0.59 ± 0.31		2.4
Reduced A chain	0.037	5	0.54 ± 0.09	10^{-3}	1.1 ± 0.3	20 ± 6	1.0
(μM)	1.0	5	15 ± 1	10^{-7}	1.7 ± 0.3	3 ± 6	1.3
	27	5	142 ± 17	10^{-7}	6.4 ± 2.9	140 ± 120	2.5
Reduced B chain	0.037	5	0.54 ± 0.04	10^{-5}	2.6 ± 0.6	35 ± 8	1.6
(μM)	1.0	5	15 ± 2	0.8 ± 0.6	2.2 ± 0.4	35 ± 8	1.9
	27	5	240 ± 87	0.4 ± 3.3	21 ± 4	26 ± 19	4.8
Reduced ribo-	0.037	5	1.8 ± 2.4	1.1 ± 2.3	0.01 ± 0.09	2 ± 3	1.1
nuclease (μM)	1.0	5	17.6 ± 0.7	10^{-7}	0.08 ± 0.01	20 ± 4	0.3
	27	5	217 ± 23	10^{-6}	0.42 ± 0.14	32 ± 21	0.65
Dihydrolipoic	0.037	6	0.55 ± 0.04	7 ± 3		10^9	7.0
acid (μM)	1.0	6	20 ± 2	20 ± 5		0.8 ± 0.2	20
	27	6	418 ± 84	144 ± 44		0.4 ± 0.1	144
2,3-Dimercapto-	0.037	6	0.70 ± 0.01	171 ± 8		1650 ± 250	171
propanol (μM)	1.0	6	19 ± 1	230 ± 20		3500 ± 400	230
	27	6	315 ± 27	490 ± 70		2200 ± 400	490
Dithiothreitol	0.037	6	0.8 ± 0.8	26 ± 45		64 ± 67	26
(μM)	1.0	6	8.1 ± 0.3	1.8 ± 0.6		1460 ± 370	1.8
	27	6	119 ± 7	9.2 ± 1.7		880 ± 220	9.2

[a] Initial velocity data were fitted with the indicated equations. Each thiol was used at 6–10 concentrations. The data given here are representative of the seven concentrations of insulin studied with each thiol. The experimental details and fitting procedure were as described in text. The apparent K_m for eq 3 and 5 is the concentration of thiol yielding half the apparent V_1 calculated from the fitted data. Note that the concentrations for GSH and mercaptoethanol are in mM; all other concentration terms are μM.

insulin as variable substrate gave linear, intersecting lines at all levels of thiol; fits to the Michaelis-Menten equation (eq 1) yielded the estimates of the parameter values given in Table I. The apparent V_m and K_m values were found to increase, and the slope to decrease, as the concentration of thiol increased, until substrate inhibition by the thiol was encountered.

The situation is much more complex when these data are examined with the thiol as variable substrate; reciprocal plots (Figure 3) are nonlinear and show definite substrate inhibition. In an attempt to distinguish the two possible explanations of the data, i.e., whether the plots are linear with superposed substrate inhibition or rather are parabolic with substrate inhibition, Hill plots of the data were made. These plots were linear over the lower levels of thiol, with slopes greater than one; while curvature was evident at the higher levels of thiol due to substrate inhibition. Fits of the Hill equation to the data on the linear portions of the Hill plot estimated the values of the Hill coefficients (mean ± standard deviation) as 1.63 ± 0.19, 1.80 ± 0.53, and 1.91 ± 0.45 for ASH, BSH, and RSH, respectively. Thus with the protein thiols there is again a necessity for a term involving the second power of the substrate concentration in an equation to describe the data, so the simplest possible description of the data (at fixed insulin concentration) is

$$v = V_1/(1 + b_1/S + b_2/S^2 + S/K_I) \quad (5)$$

Fits of eq 5 to these data are given in Table II. That this equation provides an apparently good description of the data can be seen in Figure 3; further, fits to the analogous first degree equation (eq 6) yielded overall significantly

$$v = V_1/(1 + b_1/S + S/K_I) \quad (6)$$

worse residual errors and unreasonably high values for V_1. Conversely, as can be seen in Table II, the first order term (b_1/S) in eq 5 was not important for description of the data, in keeping with the Hill coefficients near 2.0.

Non-Protein Dithiols. Dihydrolipoic acid, 2,3-dimercaptopropanol, and dithiothreitol (each with two thiol groups per molecule) were all found to be effective thiol substrates. Similar to each of the other thiols studied, reciprocal plots with insulin as substrate were linear; fits of the Michaelis-Menten equation to these data are given in Table I. While plots of these data with thiol as variable substrate appear similar to the plots for the protein thiols, application of the Hill equation to these data is not possible due to the curvature in the Hill plot caused by the substrate inhibition. Fortunately the question as to whether the reaction appeared to require one or two molecules of the thiol substrate was resolved in each case by comparison of the fits obtained with eq 5 and 6: at nearly every insulin concentration, eq 5 was eliminated by either (a) significantly worse residual errors, (b) lack of convergence to a solution, or (c) convergence to a negligible value for (b_2). Thus in contrast to the results with the protein thiols, with the low molecular weight dithiols there is no evidence for a requirement of the enzyme for more than one molecule of thiol substrate per insulin molecule.

Product Inhibition Studies. Oxidized glutathione (GSSG) was studied as an inhibitor of reaction at varying levels of insulin and of GSH. Reciprocal plots ($1/v$ vs. $1/$[insulin]) of the data at fixed levels of GSH were linear (Figure 4a and intersected on or very near the $(1/v)$ axis. Thus the possibility that GSSG is an uncompetitive (vs. insulin) inhibitor can be eliminated by inspection. To discrim-

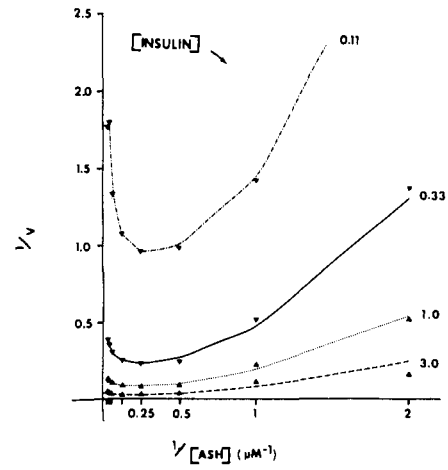


FIGURE 3: Reciprocal plots of the initial velocity of insulin degradation by GIT with reduced insulin A chain as variable substrate at fixed levels of insulin. ASH, reduced insulin A chain. The concentrations of insulin present were as indicated on the figure (in μM); v is given in picomoles of insulin degraded per minute per microgram of GIT. The lines are derived from fits of the data to eq 5. See Table II for representative fits of eq 5 to the complete data sets.

inate between competitive inhibition and noncompetitive inhibition with K_I (intercept) much greater than K_I (slope), the data at each level of GSH were fitted with both the equations for competitive and noncompetitive inhibition (eq 7 and 8, respectively). Fits to eq 8 gave values of K_{II} in the

$$v = V_1/(1 + K_m/S + K_mP/(SK_{IS})) \quad (7)$$

$$v = V_1/(1 + K_m/S + K_mP/(SK_{IS}) + P/K_{II} \quad (8)$$

range 10^6–10^8; thus there is no evidence for any effect of GSSG on the intercept and the data therefore indicate the inhibition is competitive vs. insulin (Table III).

Inspection of the plots at fixed levels of insulin (Figure 4b) eliminates the possibility that GSSG is an uncompetitive inhibitor vs. GSH. Further, the close convergence of the lines as the $(1/v)$ axis is approached shows that the inhibition is probably competitive vs. GSH. Plots of $1/v$ vs. GSSG demonstrate that this inhibition is a linear effect. Attempts to obtain comparative fits of the data to competitive and noncompetitive models are complicated by the fact that there are three possible equations for each type of inhibition. However, the possible noncompetitive equations were eliminated by either convergence to very high values of K_{II} or significantly worse residual errors (Bartfai and Mannervik, 1972a,b). Of the possible competitive equations, the closest fit to the data was given by

$$v = \frac{V_1}{\left(1 + \left(\frac{b_1}{A} + \frac{b_2}{A^2}\right)\left(1 + \frac{P}{K_{IS}}\right)\right)} \text{(competitive)} \quad (9)$$

Fits with this equation gave values for K_{IS} for GSSG of 98 ± 20, 111 ± 55, and 246 ± 79 μM at insulin concentrations of 0.33, 3.0, and 9.0 μM, respectively.

The inhibition by $ASSO_3$ and by $BSSO_3$ were qualitatively similar to that seen with GSSG; i.e., reciprocal plots with insulin as variable substrate were linear intersecting at or near the ordinate, while plots with GSH at variable substrate were parabolic converging at the ordinate. The possibility that the inhibition produced by either "product" was uncompetitive vs. either insulin or GSH could be excluded by inspection of the reciprocal plots. Further, in each case

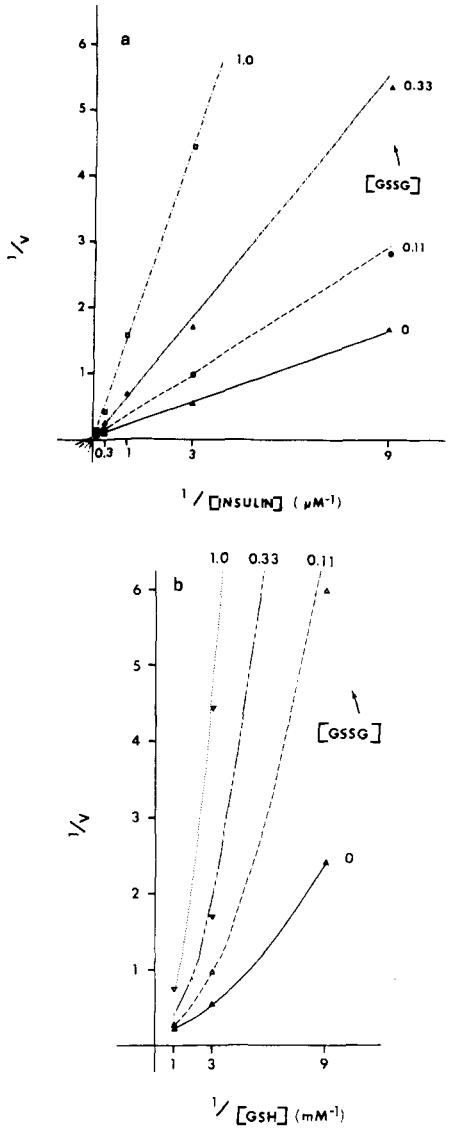


FIGURE 4: Inhibition by oxidized glutathione of insulin degradation by GIT. (a) Reciprocal plot of the initial velocity (picomoles of insulin degraded per minute per microgram of GIT) with insulin as variable substrate in the presence of 0.33 mM GSH. The concentration of GSSG present (mM) was as noted on the figure. The lines were obtained by fits to eq 7 for inhibition competitive vs. insulin (see Table III for the parameter values for the complete data set). Note that in this as well as in all the succeeding inhibition plots the fitted lines are not derived individually; e.g., in this case eq 7 was fitted to all of the data obtained with 0.33 mM GSH. (b) Reciprocal plot with GSH as variable substrate. Insulin was present at 0.33 μM; the concentration of GSSG (mM) was as noted on the figure. The lines were derived by fits of eq 9 for competitive inhibition vs. GSH.

the $1/v$ vs. $1/S$ plots appeared to converge at the ordinate, indicating that the inhibition with both S-sulfonated polypeptides was competitive vs. each substrate. That the inhibition with either S-sulfonated peptide was competitive vs. insulin was confirmed by comparison of the fits with eq 7 (Table III) and 8 as described for GSSG. Fitting the possible equations to the data with $ASSO_3$ similarly excluded the noncompetitive (vs. GSH) models by converging to very high values of K_{II}, while eq 9 could be chosen as the best model of the competitive inhibition equations on the basis of the smaller residual errors it produced. K_{IS} values for $ASSO_3$ obtained by fitting eq 9 were 6.2 ± 2.5, 9.8 ± 4.7, 24 ± 16, and 6×10^7 μM at insulin concentrations of 0.25, 2.25, 6.75, and 13.5 μM, respectively. Very large values of

Table III: Product Inhibition Studies.[a]

Inhibitor	GSH (mM)	V_1 (pmol min^{-1} μg^{-1})	K_m (insulin) (μM)	K_{IS} (μM)
GSSG	0.11	13.1 ± 3.8	10.7 ± 3.4	138 ± 22
	0.33	45.6 ± 5.1	8.2 ± 1.0	137 ± 10
	1.00	145 ± 17	10.5 ± 1.4	454 ± 33
S-sulfonated A chain	0.10	10.9 ± 7.5	3.2 ± 2.8	0.13 ± 0.12
	0.33	52.9 ± 10.1	8.7 ± 2.0	3.03 ± 0.60
	1.00	168 ± 12	10.8 ± 0.9	69 ± 32
	2.00	386 ± 21	19.3 ± 1.4	2×10^8
S-sulfonated B chain	0.11	11.8 ± 2.2	1.4 ± 0.3	1.4 ± 0.2
	0.33	20.5 ± 11.4	23.0 ± 13.8	1.3 ± 0.3
	1.00	26.5 ± 6.2	10.0 ± 2.6	0.6 ± 0.1
A chain-cysteic acid	0.20	16.4 ± 1.9	7.8 ± 1.0	78 ± 13
	1.00	127 ± 22	11.1 ± 2.1	146 ± 49
B chain-cysteic acid	0.20	13.7 ± 2.9	6.0 ± 1.5	14.3 ± 2.2
	1.00	141 ± 13	12.4 ± 1.3	25.4 ± 1.7

[a] The parameter values shown were obtained from fits of eq 7 for inhibition competitive vs. insulin. In each case the concentration of GSH was held constant as shown; the concentrations of insulin and inhibitor were varied independently. The experimental procedure and data fitting were as described in the text.

K_{IS} at the highest level of insulin indicate that there was no significant inhibition in this case, consistent with the inhibition by $ASSO_3$ being competitive vs. insulin as well as vs. GSH.

The cysteic acid derivatives of A and B chains were less effective inhibitors of insulin degradation than were the corresponding S-sulfonated forms in that much higher concentrations were necessary for measurable inhibition. Although the data with ASO_3 and BSO_3 are less extensive they clearly demonstrated that the inhibition by either derivative is not uncompetitive vs. insulin. Similarly, the intersecting nature of the $1/v$ vs. inhibitor plots with GSH as substrate excluded the possibility that either cysteic acid derivative is an uncompetitive inhibitor vs. GSH.

Discussion

Many previous studies of GIT (Katzen and Stetten, 1962; Katzen et al., 1963; Tomizawa and Varandani, 1965; Varandani and Tomizawa, 1966; Katzen and Tietze, 1966; Ansorge et al., 1973b) have used an alternate assay system, i.e., the measurement of the GSSG formed in the reaction via coupling with the oxidation of NADPH in the presence of glutathione reductase. The measurement of the change in trichloroacetic acid precipitation of ^{125}I-labeled insulin was chosen for the current study due to two reasons. The first of these is that the radioactive assay is both more sensitive and much more precise than the NADPH assay since the actual observed changes in the optical density are very small; this allows the use of less GIT and also permits the study of lower concentrations of substrates. The other consideration was that the NADPH assay permits the study of only one thiol substrate (GSH) while the radioactive assay permits the use of any thiol substrate. The basic results of the current study with GSH as substrate have been confirmed by the use of the NADPH assay: With the use of 20 μg of GIT, 2.5–100 μM insulin, and 0.5–5.0 mM GSH in a total volume of 1.4 ml, linear intersecting reciprocal plots with insulin as variable substrate were obtained, while reciprocal plots with GSH as variable substrate were parabolic (Chandler and Varandani, submitted for publication). Previous kinetic studies with GIT have not noted this nonlinearity,

probably because all of these studies were carried out only at high levels of insulin where the nonlinearity is least apparent. Since the reduction of a disulfide bond requires 2 equiv of thiol, and since the formation of the trichloroacetic acid soluble A chain requires the disruption of both of the interchain disulfide bonds of insulin, the finding of parabolic reciprocal plots and Hill coefficients greater than one for the thiol substrates is not surprising.

The possibility that GIT might follow a ping-pong mechanism (i.e., that the enzyme is reduced by GSH with the reduced enzyme form then reacting with insulin) was considered because of three previous findings. (a) The native purified enzyme is not affected by *N*-ethylmaleimide or iodoacetate, while after prior incubation with GSH these sulfhydryl blocking reagents completely abolish the activity of the enzyme (Varandani and Plumley, 1968; Varandani and Nafz, 1969); (b) the purified enzyme has been found not to bind [^{14}C]iodoacetate whereas after reaction with GSH the enzyme sequesters [^{14}C]iodoacetate (Varandani and Nafz, 1969); and (c) spectrometric evidence for the formation of reduced GIT by a reaction with GSH in the absence of disulfide substrate (insulin) has been reported (Katzen and Tietze, 1966). These findings suggested the possibility of two enzymic forms: an oxidized disulfide form (native enzyme) and a reduced form containing an accessible thiol group formed after reaction with GSH. However, the present results are inconsistent with a ping-pong mechanism, since the basic reciprocal plots of initial velocity should consist of a series of parallel lines for this type mechanism (Cleland, 1963a). This result indicates that cyclic reduction and oxidation of the enzyme is kinetically not detectable; therefore the additions of GSH and of insulin are not separated by a product release step in the reaction sequence. In this case the enzyme would not exhibit a ping-pong mechanism, although it could still undergo repeated oxidation–reduction.

The results of the product inhibition studies may be summarized as follows: all inhibition effects were linear; GSSG was competitive vs. both insulin and GSH; $ASSO_3$ was competitive vs. both substrates; $BSSO_3$ was competitive vs. insulin, and either competitive or noncompetitive vs. GSH; and neither ASO_3 nor BSO_3 was uncompetitive vs. either substrate. Of the products used, GSSG is the only product which was in the correct chemical form. The A and B chains are the GIT-catalyzed degradation products arising from insulin, and are probably released in the reduced (-SH) form (Tomizawa, 1962; Katzen and Stetten, 1962; Tomizawa and Varandani, 1965; Varandani and Tomizawa, 1966). Although B chain is usually found in a disulfide-linked aggregate with a small amount of A chain (Varandani et al., 1972; Varandani, 1973b,d), this aggregate is probably formed subsequent to release of B chain from GIT (Katzen and Stetten, 1962; Varandani et al., 1972). Since the A and B chains are separated by GIT, both of the interchain disulfide bridges must be split in the course of the reaction. On the basis of the stoichiometry of the reaction, all three disulfide bonds of insulin appear to be susceptible to reduction in the presence of excess GSH (Katzen et al., 1963). Whether the two interchain bonds are split preferentially or the three disulfide bonds are split in any specific order is not known. Thus, the actual products formed from insulin may be completely reduced A and B chains, these peptides with internal disulfide bonds, or aggregates with interchain disulfide bonds. Unfortunately, none of the above derivatives is suitable for product inhibition studies: the reduced forms can serve at low concentrations as thiol substrates in place of GSH; and attempts to prepare the disulfide containing forms from the reduced chains yield preparations containing residual thiol-containing peptides or aggregated disulfide forms of limited solubility. Therefore the relatively stable S-sulfonated and cysteic acid derivatives were used. The cysteic acid derivatives must be "dead end" inhibitors, since this group cannot be altered under these conditions. The S-sulfonated peptides might behave as true products (i.e., participate in the reverse reaction), however, since the ($-SO_3$) moiety may be removed by reduction with GSH.

For a reaction with three substrates (i.e., insulin and two molecules of thiol substrate) and three products (A chain, B chain, and GSSG), it can be predicted (Cleland, 1963c) that for an "ordered" mechanism (Scheme II) one product

Scheme II

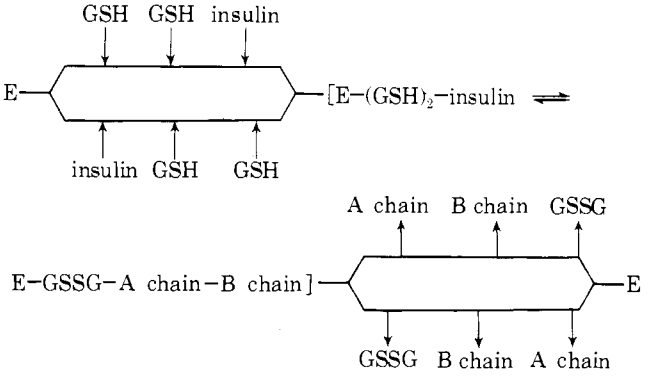
$$E \xrightarrow{A} EA \xrightarrow{B} EAB \xrightarrow{C} (EABC \rightleftharpoons$$

$$EPQR) \xrightarrow{P} EQR \xrightarrow{Q} ER \xrightarrow{R} E$$

(Q) will be uncompetitive inhibitor vs. each substrate (assuming the other products are absent) and also that no product can be competitive vs. every substrate (at nonsaturating levels of the other substrates). The present data are clearly in conflict with each of these predictions, since uncompetitive inhibition was not observed with any inhibitor–substrate pair, and two products, GSSG and $ASSO_3$, were shown to be competitive inhibitors vs. both GSH and insulin; this indicates that an "ordered" mechanism is not operative.

Competitive inhibition is shown by an inhibitor which reacts only with same enzyme species as does the competing substrate (Cleland, 1963b,c). From this it follows that competitive inhibition vs. two substrates, as shown by GSSG and $ASSO_3$, requires that both substrates react with the same enzyme species; therefore, the reaction must have alternate possible sequences for the addition of substrates. Similarly, since all of the products tested (GSSG, $ASSO_3$, and $BSSO_3$) showed competitive inhibition, the order of release of products must also have alternate possible pathways. This conclusion is supported by the findings of "slope" effects with the dead-end inhibitors (ASO_3 and BSO_3), inasmuch as the slope of a reciprocal plot can be affected by a dead-end inhibitor only when it reacts with the same enzyme form as does the varied substrate (Cleland, 1967). A mechanism consistent with the kinetic data and with a random mechanism for GIT is presented in Scheme III.

Scheme III: Possible Mechanism for the Degradation of Insulin by GIT.



The nature of the thiol substrate has little effect on the velocity of the reaction when the thiol concentration is extrapolated to infinity (compare the V_1 values at the various insulin concentrations in Table II). In most cases the apparent difference from the average V_1 values for all thiol substrates were less than 2, and much of these differences may be due to uncontrolled interexperimental variations. Thus the apparent K_m values for the thiols can be taken as a measure of the relative effectiveness of the thiols as substrate. This ranking is: reduced ribonuclease > dithiothreitol > reduced A chain > reduced B chain > dihydrolipoic acid > 2,3-dimercaptopropanol > GSH > mercaptoethanol.

There are clearly differences among the protein polythiols (with Hill coefficients near 2 with substrate inhibition), the non-protein dithiols (Hill coefficients close to 1.0 with substrate inhibition) and the monothiols mercaptoethanol and glutathione (Hill coefficients intermediate between 1 and 2, with no substrate inhibition). Since the reduction of a disulfide bond requires 2 equiv of thiol, the interpretation of a Hill coefficient greater than 1 as indicating the utilization of two molecules of substrate is reasonable in the case of monothiols such as GSH or mercaptoethanol. However, the necessary 2 equiv of thiol might be supplied by a single molecule which contains two or more thiol groups, provided it can form an internal disulfide bond; evidence for this occurrence in the present case is provided by the apparent requirement for only a single molecule of dithiothreitol, dihydrolipoic acid, or dimercaptopropanol. Furthermore, a Hill coefficient greater than 1 can be found for a substrate in a random mechanism even though only 1 equiv of the substrate is required (Fisher, 1972). The substrate inhibition by the di- and protein thiols is also consistent with a random mechanism (Fisher, 1972). There are, however, other possible explanations of this substrate inhibition (Webb, 1963; Cleland, 1967). In view of the macromolecular size of the substrates, it is probable that GIT has multiple sites (i.e., an insulin-binding site and a thiol binding site (s)); hence the substrate inhibition could be due to an interaction of the thiol substrate with the insulin site which interferes with insulin binding. Alternatively, the enzyme might act via an allosteric (cooperative) mechanism. Another possibility is that the thiol substrates are contaminated with the disulfide form of the substrate, which might act as a product inhibitor. While such contamination is possible in view of the well-known lability of thiol groups in solution, such a contaminant might be expected to behave similarly to GSSG as a competitive inhibitor vs. both substrates. Contamination of substrate with a competitive inhibitor should not lead to the observation of substrate inhibition, however (Cleland et al., 1973).

In conclusion, both the initial velocity patterns with alternate thiol substrates and the results of inhibition studies with products and dead-end inhibitors are most consistent with a random mechanism for GIT.

Acknowledgment

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References

Ansorge, S., Bohley, P., Kirschke, H., Langner, J., Marquardt, I. Wiederanders, B., and Hanson, H. (1973a), *FEBS Lett. 37,* 238.

Ansorge, S., Bohley, P., Kirschke, H., Langner, J., Wiederanders, B., and Hanson, H. (1973b), *Eur. J. Biochem. 32,* 27.

Askelof, P., Axelsson, K., Eriksson, S., and Mannervik, B. (1974), *FEBS Lett. 38,* 263.

Bartfai, T., and Mannervik, B. (1972a), *FEBS Lett. 26,* 252.

Bartfai, T., and Mannervik, B. (1972b), Analysis and Simulation, Amsterdam, North-Holland Publishing Co., p 197.

Chandler, M. L., and Varandani, P. T. (1972), *Biochim. Biophys. Acta 286,* 136.

Chandler, M. L., and Varandani, P. T. (1973a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 299.

Chandler, M. L., and Varandani, P. T. (1973b), *Biochim. Biophys. Acta 320,* 258.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67,* 104.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67,* 173.

Cleland, W. W. (1963c), *Biochim. Biophys. Acta 67,* 188.

Cleland, W. W. (1967), *Annu. Rev. Biochem. 36,* 77.

Cleland, W. W., Gross, M., and Folk, J. E. (1973), *J. Biol. Chem. 248,* 6541.

Dammkoehler, R. A. (1966), *J. Biol. Chem. 241,* 1955.

Daniel, C., and Wood, F. S. (1971), Fitting Equations to Data, New York, N.Y., Wiley-Interscience.

Fisher, J. R. (1972), *Arch. Biochem. Biophys. 152,* 638.

Fuchs, S. Delorenzo, F., and Anfinsen, C. B. (1967), *J. Biol. Chem. 242,* 298.

Goldberger, R. T., Epstein, C. J., and Anfinsen, C. B. (1963), *J. Biol. Chem. 238,* 628.

Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 197.

Katzen, H. M., and Stetten, D., Jr. (1962), *Diabetes 11,* 271.

Katzen, H. M., and Tietze, F. (1966), *J. Biol. Chem. 241,* 3561.

Katzen, H. M., Tietze, F., and Stetten, D. (1963), *J. Biol. Chem. 238,* 1006.

Marquardt, D. W. (1963), *J. Soc. Ind. Appl. Math. 11,* 431.

Narahara, H. T., and Williams, R. H. (1959), *J. Biol. Chem. 234,* 71.

Payne, R. B. (1972), *Clin. Chim. Acta 42,* 263.

Spolter, P., and Vogel, J. (1968), *Biochim. Biophys. Acta 167,* 525.

Thomas, J. H., and Wakefield, S. M. (1973), *Biochem. Soc. Trans. 1,* 1179.

Tomizawa, H. H. (1962), *J. Biol. Chem. 237,* 428.

Tomizawa, H. H., and Halsey, Y. D. (1959), *J. Biol. Chem. 234,* 307.

Tomizawa, H. H., and Varandani, P. T. (1965), *J. Biol. Chem. 240,* 3191.

Varandani, P. T. (1966a), *Biochim. Biophys. Acta 118,* 198

Varandani, P. T. (1966b), *Biochim. Biophys. Acta 127,* 246.

Varandani, P. T. (1967), *Biochemistry 6,* 100.

Varandani, P. T. (1968), *Diabetes 17,* 547.

Varandani, P. T. (1972), *Biochim. Biophys. Acta 286,* 126.

Varandani, P. T. (1973a), *Biochem. Biophys. Res. Commun. 55,* 689.

Varandani, P. T. (1973b), *Biochim. Biophys. Acta 295,* 630.

Varandani, P. T. (1973c), *Biochim. Biophys. Acta 304,* 642.
Varandani, P. T. (1973d), *Biochim. Biophys. Acta 320,* 249.
Varandani, P. T. (1974a), *Diabetes 23,* 117.
Varandani, P. T. (1974b), *Biochim. Biophys. Acta 371,* 577.
Varandani, P. T., and Nafz, M. A. (1969), *Diabetes 18,* 176.
Varandani, P. T. and Nafz, M. A. (1970a), *Int. J. Biochem. 1,* 313.
Varandani, P. T., and Nafz, M. A. (1970b), *Arch. Biochem. Biophys. 141,* 553.
Varandani, P. T., Nafz, M. A., and Chandler, M. L. (1975), *Biochemistry,* following paper in this issue.
Varandani, P. T., Nafz, M. A., and Shroyer, L. A. (1971), *Diabetes 20,* 342.
Varandani, P. T., and Plumley, H. (1968), *Biochim. Biophys. Acta 151,* 273.
Varandani, P. T., Shroyer, L. A., and Nafz, M. A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1681.
Varandani, P. T., and Tomizawa, H. H. (1966), *Biochim. Biophys. Acta 113,* 498.
Venetianer, P., and Straub, F. B. (1963), *Biochim. Biophys. Acta 67,* 166.
Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. 1, New York, N.Y., Academic Press, p 111.
Wilkinson, G. N. (1961), *Biochem. J. 80,* 324.

Interaction of Insulin Analogs, Glucagon, Growth Hormone, Vasopressin, Oxytocin, and Scrambled Forms of Ribonuclease and Lysozyme with Glutathione–Insulin Transhydrogenase (Thiol:Protein-Disulfide Oxidoreductase): Dependence upon Conformation†

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ABSTRACT: Interactions of several proteins with glutathione–insulin transhydrogenase (GIT) have been investigated by determining their ability to inhibit degradation of ^{125}I-labeled insulin catalyzed by GIT. The inhibition by every insulin analog (des-Asn-des-Ala-pork insulin, desoctapeptide-pork insulin, des-Ala-pork insulin, pork insulin, proinsulin, and guinea pig insulin) was competitive vs. insulin indicating that they function as alternate substrates. The insulin analogs with the least hormonal activity showed the highest potency as inhibitors of insulin degradation. Whereas native ribonuclease and lysozyme showed little or no inhibition, their scrambled forms (i.e., reduced and randomly reoxidized) showed competitive inhibition with a potency greater than that of insulin. These results suggest that the conformation of the substrate or inhibitor is probably the major factor in determining the specificity for (or binding to) the enzyme. Studies with other peptide hormones showed competitive inhibition with vasopressin and oxytocin and noncompetitive inhibition with glucagon. The inhibition with growth hormone could be either competitive or noncompetitive. The inhibition by glucagon and growth hormone (physiologic antagonists of insulin) could serve as a control mechanism to modulate the activity of enzyme. The following showed very little or no inhibition: the native and scrambled form of pepsinogen, trypsin inhibitor of beef pancreas and of lima bean, C-peptide of pork proinsulin, and heptapeptide (B_{23}–B_{29}) of insulin.

In a companion publication we reported (Chandler and Varandani, 1975) on the kinetic mechanism of insulin degradation by glutathione–insulin transhydrogenase (GIT,[1] thiol:protein-disulfide oxidoreductase, EC 1.8.4.2). In addition to degrading insulin, GIT under appropriate in vitro conditions is capable of performing other activities; i.e., the inactivation of proinsulin (Varandani and Nafz, 1970), the reactivation of reduced (Katzen and Tietze, 1966) and scrambled (i.e., reduced and randomly reoxidized) (Ansorge et al., 1973; Varandani, 1974b) ribonuclease, the reactivation of scrambled proinsulin (Varandani and Nafz, 1970), the reactivation of reduced insulin (Katzen et al., 1963), the regeneration of insulin from reduced A and B chains (Varandani, 1967), and the reduction of insulin derivatives (Katzen and Tietze, 1966) and of oxytocin and vasopressin (Katzen and Stetten, 1962).

In the present paper, inhibition of GIT-catalyzed insulin degradation by several proteins, which are insulin analogs

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[1] Abbreviation used is: GIT, glutathione–insulin transhydrogenase. As noted in the accompanying paper, we have retained the designation glutathione–insulin transhydrogenase for consistency with previous publications.