Effect of Protein and Peptide Inhibitors on the Activity of Protein Disulfide Isomerase†

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ABSTRACT: The protein disulfide isomerase catalyzed reduction of insulin by glutathione is inhibited by peptides of various length and amino acid composition. Peptide inhibitors are competitive against insulin and noncompetitive against GSH, consistent with a sequential rather than a double displacement mechanism. Peptides of unrelated primary sequence that do not contain cysteine inhibit the GSH–insulin transhydrogenase activity of PDI, and the affinity of these peptides toward the enzyme is largely dependent on the peptide length rather than composition, hydrophobicity, or charge. Cysteine-containing peptides are 4–8-fold better inhibitors than non-cysteine-containing peptides of the same length, suggesting a cysteine-specific component to the interaction with the enzyme. Oxidized insulin chain B also inhibits the oxidative folding of reduced ribonuclease in a glutathione redox buffer with an inhibition constant that is comparable to that observed for the inhibition of insulin reduction, suggesting a similar if not identical binding site for the catalysis of oxidative protein folding and the reduction of insulin.

Protein disulfide isomerase (PDI,[1] EC 5.3.4.1) was discovered in the search for a physiological catalyst for the oxidation and folding of reduced ribonuclease to its native conformation (Goldberger et al., 1963; Venetianer & Straub, 1963). The enzyme has been detected in most vertebrate tissues and shown to be a homodimer consisting of subunits of a molecular weight of 57000 (Hillson et al., 1984). PDI is thought to be important in catalyzing thiol/disulfide exchange reactions which occur in the posttranslational formation of disulfide bonds in newly synthesized proteins (Freedman et al., 1988; Freedman, 1984; Creighton et al., 1980). PDI accepts a wide variety of substrates ranging from single and multidomain proteins to low molecular weight thiols and disulfides (Freedman et al., 1984; Koivu & Myllylä, 1987; Morin & Dixon, 1985).

PDI also catalyzes the reductive cleavage of insulin by GSH, a reaction which has been suggested to have physiological importance in the degradation of insulin (Dowson & Varandani, 1987; Duckworth, 1988; Varandani, 1978). It has been proposed that during catalysis the enzyme undergoes reduction by GSH and the reduced form of the enzyme is an active intermediate during transhydrogenase-catalyzed reduction of disulfides (Katzen & Tietze, 1966). In addition, PDI catalyzes the formation of native insulin from scrambled insulin with randomly joined disulfide bonds and from the separate A and B chains of insulin through thiol/disulfide exchange (Tang et al., 1988).

Protein disulfide isomerase has been cloned and sequenced from rat and human (Edman et al., 1985; Pihlajaniemi et al., 1987). Two different regions of the PDI primary sequence are homologous to the active-site region of thioredoxin, a redox-active dithiol/disulfide-containing protein (Freedman et al., 1988; Holmgren, 1989). The enzyme also displays thyroid hormone binding activity (Yamauchi et al., 1987; Cheng et al., 1987), serves as the β-subunit of prolyl 4-hydroxylase (Koivu et al., 1987; Pihlajaniemi et al., 1987), and may be involved as a glycosylation site binding protein of glucosyl transferase of the endoplasmic reticulum (Geetha-Habib et al., 1988). PDI has recently been shown to copurify as a component of the microsomal triglyceride transfer complex (Wetterau et al., 1990) and to catalyze the GSH-dependent reduction of dehydro-L-ascorbate (Wells et al., 1990).

An essential disulfide/dithiol group has been implicated in catalysis by the inhibition of the enzyme by cadmium chloride and sodium arsenite, (Hillson & Freedman, 1980; Kurup et al., 1966) and by inactivation with alkylating agents after incubation of the enzyme with a reducing reagent (Lambert & Freedman, 1984; Hawkins et al., 1976; Gilbert, 1989). The kinetic mechanism of PDI is unclear. Lambert and Freedman (1983b) mentioned that the reaction of PDI might involve a ternary complex. Recently, Gilbert (1989) suggested a hybrid sequential ping-pong mechanism for the reduction of a synthetic disulfide containing peptide (CYIQNC) by GSH. However, at the highest attainable substrate concentrations, saturation behavior was not observed for the PDI-catalyzed reduction of CYIQNC by GSH.

In this work, we use the glutathione–insulin transhydrogenase activity of PDI and the PDI-catalyzed oxidative renaturation of fully reduced RNase to explore and quantitate the specificity of the interaction of the protein with peptides of various sequences and lengths.

Experimental Procedures

Materials. Glutathione (GSH), glutathione disulfide (GSSG), insulin (bovine pancreas), insulin chain B (oxidized, sulfonated, from bovine insulin), insulin chain A (oxidized, sulfonated, from bovine insulin), stomatostatin, glucagon, AGCKNNFFWKTFTSC, CYIQNC (tocinoic acid), hexaglycine, TGGFL, VGDE, GHK, cCMP, ribonuclease A, and glutathione reductase (from bakers' yeast, type III) were purchased from Sigma Chemical Co. Reductacryl reagent was obtained from CalBiochem. Dithiothreitol was purchased from Boehringer Mannheim Biochemicals. Angiotensin I

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[1] Abbreviations: PDI, protein disulfide-isomerase; GSH, glutathione; GSSG, glutathione disulfide; RNAse, bovine pancreatic ribonuclease A; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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(human) was obtained from Vega Biochemical. The materials for SDS–polyacrylamide gel electrophoresis were obtained from Bio-rad. The peptide ERYLKDQQLLGIWGCSGKLG was a generous gift from Professor M. Z. Atassi, Department of Biochemistry, Baylor College of Medicine, Houston, TX. The peptides SLSAGDESDALGK and TFLKVLKGDKSAGGKVLKSGKN were generous gifts from Dr. Fernando Monroy and Dr. M. H. Dresden, Department of Biochemistry, Baylor College of Medicine, Houston, TX, and the peptide YEQLDEEHKKIFKGIGDCTRD was a generous gift from Dr. Peter E. Wright of the Department of Molecular Biology, Scripps Clinic, LaJolla, CA. All commercial peptides used in this work were 95–99% pure. The noncommercial peptides were purified by gel filtration and were at least 93% pure as judged by HPLC (linear gradient 0.1% TFA to 0.1% TFA in 70% acetonitrile over 60 min with detection by absorbance at 220 nm) except for ERYLKDQQLLGIWGCSGKLG which was 86% pure. The impurities in this peptide were distributed among several species each of which comprised <5% of the total. Cysteine-containing peptides were prereduced by incubation at pH 7.5 with 10 mM GSH for 5–10 min or by incubation with excess reductacryl. Performic acid oxidation of the peptide ERYLKDQQLLGIWGCSGKLG was performed by the method of Hirs (1967). The peptide was dialyzed (Spectrapor 7, MW cutoff 1000) before use. HPLC of the performic acid oxidized peptide showed a single major peak (>85%) which eluted significantly earlier than the cysteine-containing peptide from which it was derived. Impurities in the performic acid oxidized peptide consisted of multiple minor species all of which represented less than 5% of the major peak. The performic acid oxidized peptide contained less than 2% of the starting cysteine-containing peptide. All other chemicals were of high purity. Glass-distilled, deionized water was used for all experiments.

Protein disulfide isomerase was prepared from bovine liver by the method of Lambert and Freedman (1983a). The purity of the enzyme was greater than 95% as judged by SDS–polyacrylamide gel electrophoresis. The enzyme was stored at −20 °C in 20 mM sodium phosphate buffer, pH 6.3.

Enzyme Assay and Kinetic Measurements. The PDI-catalyzed reduction of insulin by GSH was monitored by coupling the reduction of the product, GSSG, to NADPH oxidation with glutathione reductase (Lambert & Freedman, 1983b; Chandler & Varandani, 1975). The enzyme activity, in the coupled reaction, was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm using a Beckman DU-7 or DU-70 spectrophotometer with the cells maintained at 25 °C. Reaction mixtures contained 0.2 M potassium phosphate buffer, pH 7.5, 5 mM EDTA, 0.15 mM NADPH, 16 units of glutathione reductase, 0.48 μM PDI, and appropriate concentrations of GSH and insulin in a total volume of 0.4 or 1.0 mL. Glutathione, NADPH, glutathione reductase, and the peptide inhibitors were incubated for at least 2 min in 0.2 M potassium phosphate, pH 7.5, containing 5 mM EDTA. Insulin was added, and the rate of nonenzymatic reduction of insulin was monitored for 2 min. Background rates in the absence of PDI were less than 0.01 absorbance unit/min at the highest GSH concentration used. The activity was measured after the addition of PDI and corrected for the noncatalyzed reaction. Initial rates were measured at least in duplicate, and the absorbance change at 340 nm was linear within the time course of the assay. One enzyme unit is defined as that amount of enzyme which catalyzes the formation of 1 μmol of GSSG/min. At high concentrations of GSH (>20 mM), the uncatalyzed reduction of insulin consumed a significant fraction of the substrate (>10%), limiting the maximum concentration of GSH which could be used.

The concentration of NADPH was determined by using $\epsilon_{340} = 6.23 \times 10^3\ M^{-1}\ cm^{-1}$. The concentration of insulin was determined, in 25 mM potassium phosphate buffer, pH 7, at 280 nm ($\epsilon = 6080\ M^{-1}\ cm^{-1}$) (Kirschenbaum, 1971). The concentration of cCMP was determined at 296 nm ($\epsilon = 0.19\ mM^{-1}\ cm^{-1}$) (Lyles & Gilbert, 1990), and the GSH concentration was determined by the method of Ellman (1959). The PDI concentration was estimated by using $E_{0.1\%} = 0.94$ at 280 nm (Gilbert, 1989).

The activity of PDI in the oxidative renaturation of RNase was determined at 25.0 °C by a continuous assay as previously described (Lyles & Gilbert, 1990). Fully reduced RNase (4–8 μM final concentration) was added to a cuvette containing buffer (0.1 M Tris–acetate/2 mM EDTA, pH 8.0), 1.0 mM GSH, 0.2 mM GSSG, the appropriate concentration of peptide, 1.1 μM PDI, and 4.5 mM cCMP, a substrate for active RNase. PDI activity was measured by the increase in RNase activity with time. Fully reduced RNase was prepared as previously described (Lyles & Gilbert, 1990). The reduced RNase contained 7.5 ± 0.5 mol of SH/mol of protein. RNase concentration was determined by the absorbance at 277.5 nm using an extinction coefficient of 9.3 $mM^{-1}\ cm^{-1}$ for the reduced enzyme (Schaffer et al., 1975).

Data Analysis. Initial velocity kinetic data were fit to the appropriate kinetic models by unweighted nonlinear least squares (Bevington, 1969). Competitive inhibitors were fit to eq 1, and noncompetitive inhibition was evaluated according to eq 2 (Cleland, 1970) where [I] is the concentration of

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_{is}}\right)\frac{1}{[S]} \quad (1)$$

$$\frac{1}{v} = \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_{ii}}\right) + \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_{is}}\right)\frac{1}{[S]} \quad (2)$$

inhibitor, [S] is the concentration of the variable substrate (GSH, insulin, or reduced RNase), K_m and V_{max} represent the apparent Michaelis constant and V_{max} observed at the concentration of the nonvariable substrate used in the experiment, and K_{ii} and K_{is} are the intercept and slope inhibition constants, respectively. Alternative models were considered for each inhibitor, and the model yielding the lowest residual with the coefficients of the lowest standard deviations was used.

The initial velocity of insulin reduction by GSH was fit by a rapid equilibrium random kinetic model (eq 3) which in-

$$v = \{V_{max}[S][G]\}/\{K_S[G] + K_G[S] + K_SK_G + [S][G] + K_S[G]([G] + K_G/K_{ig})/K_{ig}\} \quad (3)$$

cludes terms for competitive substrate inhibition by GSH due to the binding of GSH at the insulin binding site in competition with insulin (Segel, 1975) where [S] represents the concentration of the substrate, insulin, [G] represents the concentration of GSH, V_{max} is the maximum velocity, K_G and K_S are the K_m's for GSH and insulin, respectively, and K_{ig} is the inhibition constant for the substrate inhibition by GSH. In deriving this simplified version of the equation presented by Segel (1975), the equilibrium dissociation constants for GSH binding to the free enzyme and to the enzyme–insulin complex were assumed to be equal (K_G) as were the equilibrium dissociation constants for insulin binding to the free enzyme and to the enzyme–GSH complex. Inhibitory binding of GSH to the insulin site (K_{ig}) was also assumed to be independent of the occupancy of the GSH site by GSH. More complex kinetic

Table I: Inhibition Constants for Peptide Inhibition of the Glutathione–Insulin Transhydrogenase Activity of Protein Disulfide Isomerase

peptide	length (residues)	max inhibitor concn (μM)	K_{obs}[a] (μM)
insulin chain B, FVNQHLC(SO_3^-)GSHLVEALYLVC(SO_3^-)GERGFFYTPKA	30	200	200 ± 40
glucagon, HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	29	200	200 ± 50
TFLKVLKGDKSAGGKVLKSGKN	22	500	370 ± 60
insulin chain A, GIVEQC(SO_3^-)C(SO_3^-)ASVC(SO_3^-)SLYQLENYC(SO_3^-)N	21	400	380 ± 100
ERYLKDQQLLGIWGC(SO_3^-)SGKLG	20	200	240 ± 30
SLSAGDESDALGK	13	300	619 ± 44
angiotensin I, DRVYIHPFHL	10	600	660 ± 40
GGGGGG	6	5000	8500 ± 3500
YGGFL	5	3000	1300 ± 110
YGDE	4	10000	>5000
GHK	3	25000	>10000
cysteine-containing peptides			
YEQLDEEHKKIFKGIFDCIRD	21	300	96 ± 22
ERYLKDQQLLGIWGCSGKLG	20	100	46 ± 9
stomatostatin, AGCKNFFWKTFTSC	14	200	79 ± 12
tocinoic acid, CYIQNC	6	500	280 ± 60
glutathione	3	20000	4400 ± 1600

[a] Observed inhibition constants were determined by nonlinear least-squares fitting to eq 1 using at least five different inhibitor concentrations and two to five different insulin concentrations with at least duplicate determinations of individual velocity measurements. The indicated errors are standard deviations of the parameter values.

models in which the dissociation constants for substrate and inhibitor depend on the occupancy of the other substrate sites or which involve an ordered sequential addition of substrates where GSH acts as a competitive inhibitor of insulin binding will also fit the observed kinetic data. However, these models are more complex than eq 3. Consequently, the data were fit to the simpler model of eq 3.

Results

Initial Velocity Studies. Double-reciprocal plots of the initial velocity of the PDI-catalyzed reduction of insulin against GSH concentration show a family of straight lines of different slopes at low GSH concentrations (<10 mM) (Figure 1) with significant substrate inhibition at higher GSH concentrations. At low GSH concentrations (<10 mM), where substrate inhibition is not apparent, replots of the slopes and intercepts against the reciprocal of the insulin concentration are linear (not shown). At higher GSH concentrations, the velocity decreases, introducing curvature that is characteristic of substrate inhibition by GSH. At higher insulin concentrations, the substrate inhibition by GSH is diminished. The entire data set can be described by a rapid equilibrium random sequential mechanism which includes terms for competitive substrate inhibition by GSH binding to the insulin site (eq 3). More complex models such as an ordered sequential mechanism will also describe the data equally well. The K_m's for insulin and GSH are 5.3 ± 1.2 μM and 17 ± 4 mM, respectively (eq 3). The inhibition constant for substrate inhibition by GSH is 4.4 ± 1.6 mM (Figure 1).

Inhibition Studies. The PDI-catalyzed reduction of insulin by GSH is inhibited by peptides. At a fixed GSH concentration (10 mM), the peptides display linear competitive inhibition with insulin as the variable substrate. At fixed concentrations of insulin (8 μM), the peptides display noncompetitive inhibition against GSH as the variable substrate (Figure 2). Slope and intercept replots of the data in Figure 2 against insulin chain B concentration are both linear (not shown). The K_i values for a number of peptides are compiled in Table I.

Peptides that contain cysteine residues are approximately 4–8-fold better inhibitors than other peptides of comparable length (Table I). The inhibition by cysteine-containing peptides could result from the peptides serving as alternate substrates that consume insulin in a GSH-independent fashion (Chandler & Varandani, 1975) and inhibit the formation of

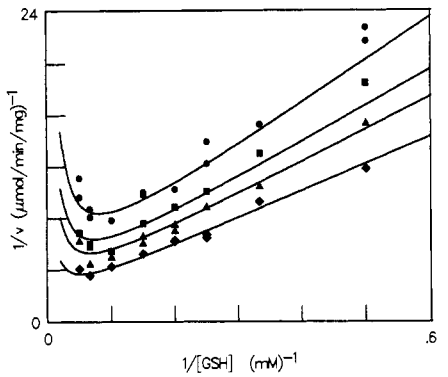

FIGURE 1: Dependence of the initial velocity of PDI-catalyzed reduction of insulin by GSH on the concentrations of insulin and GSH. The insulin concentrations were 5.3 (●), 7.9 (■), 10.6 (▲), and 21.1 μM (♦). The experiments were performed in 0.2 M potassium phosphate buffer, pH 7.5, and 5 mM EDTA at 25 °C. The solid lines are drawn according to eq 3 using V_{max} = 1.0 ± 0.16 unit/mg, K_S = 5.3 ± 1.2 μM, K_G = 17 ± 4 mM, and K_{ig} = 4.4 ± 1.6 mM.

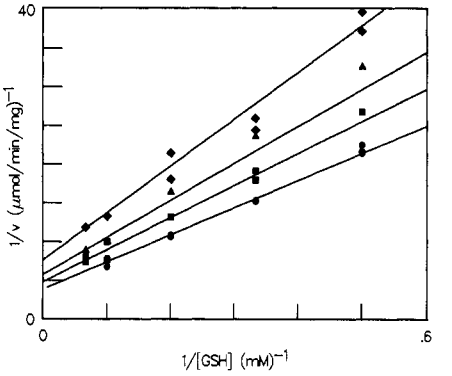

FIGURE 2: Inhibition of the glutathione–insulin transhydrogenase activity of PDI by insulin chain B (sulfonic acid) with GSH as the variable substrate. The concentration of insulin was 8 μM (nonsaturating), and the insulin chain B concentrations were 0 (●), 50 (■), 100 (♦), and 200 μM (▼). Curves are drawn by using eq 2 with an apparent V_{max} of 0.26 ± 0.02 μmol min^{-1} mg^{-1}, an apparent K_m for GSH of 9 ± 1 mM, a slope inhibition constant (K_{is}) of 270 ± 70 μM, and an intercept inhibition constant (K_{ii}) of 200 ± 60 μM.

GSSG. However, at a 10 mM concentration of GSH, the cysteine-containing peptides (at their highest concentration) did not increase the overall rate of insulin reduction as monitored by the increased turbidity resulting from precipitation of insoluble chain B that begins about 5 min after the 2-min

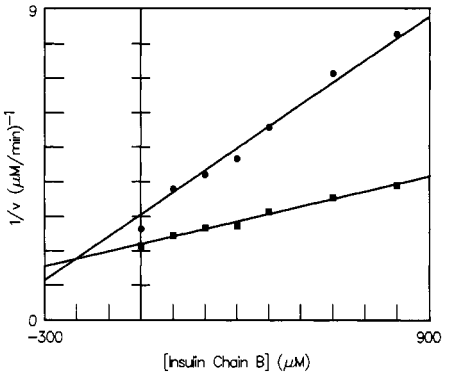

FIGURE 3: Dixon plot of the inhibition of the PDI-catalyzed oxidative renaturation of RNase by insulin chain B (sulfonic acid). The rate of RNase activation was determined as described by Lyles and Gilbert (1990). The reaction was performed in 0.1 M Tris–acetate buffer, pH 8.0, containing 2 mM EDTA, 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, 1.1 μM PDI, and ribonuclease and insulin chain B at the indicated concentrations. The concentrations of RNase were 4.0 (●) and 8 μM (■). The inhibition constant for insulin chain B (sulfonic acid) is 230 ± 40 μM.

standard assay (Holmgren, 1979). With concentrations of insulin near the K_m, inclusion of cysteine-containing peptides in the assay did not introduce additional curvature; the peptides did not cause significant depletion of the insulin substrate. The cysteine-containing peptides are also competitive inhibitors against insulin, suggesting that the inhibition arises by combination of the peptides with the enzyme at the insulin binding site rather than by substrate depletion or action as alternative substrates. However, given the complexity of the thiol/disulfide exchange reaction catalyzed by PDI (Gilbert, 1989), it is not possible to rigorously exclude all possible alternative mechanisms of inhibition by cysteine-containing peptides.

Oxidized insulin chain B is also an inhibitor of the PDI-catalyzed oxidative renaturation of fully reduced RNase. A Dixon plot (Figure 3) at two different levels of RNase indicates that the inhibition is competitive. The inhibition constant (230 ± 40 μM) is identical within experimental error with that determined by the inhibition of insulin reduction.

Discussion

Initial velocity studies suggest that the mechanism for the PDI-catalyzed reduction of insulin by GSH is a sequential kinetic mechanism involving a ternary complex rather than a double-displacement or ping-pong mechanism (Cleland, 1963, 1970). Lambert and Freedman (1983b) have mentioned a mechanism for PDI involving formation of a ternary complex, but no data were reported. Chandler and Varandani (1975) have also reported similar results at lower GSH concentrations using the increase in acid-precipitable radioactivity to follow the reduction of insulin. A hybrid mechanism for PDI-catalyzed reduction of the peptide CYIQNC by GSH has been proposed (Gilbert, 1989). This mechanism has characteristics of a sequential order of addition at high GSH concentrations and characteristics of a ping-pong mechanism at low concentrations of GSH. In this study, an intersecting family of lines was obtained, suggesting a sequential addition of insulin and GSH to PDI (Figure 1) at least at GSH concentrations less than 10 mM. The sequential mechanism observed from the initial velocity pattern at low concentrations of GSH could result from an ordered or random addition of GSH and insulin to PDI. Equations based either on a rapid equilibrium random mechanism (eq 3) or on an ordered sequential mechanism adequately describe the observed dependence of the rate on GSH and insulin concentrations. However, the initial velocity kinetic experiments are inconsistent with a ping-pong or binary complex mechanism.

The substrate inhibition observed at high concentrations of GSH is reversed by high concentrations of insulin. This is characteristic of competitive substrate inhibition by GSH (Segel, 1975). Competitive substrate inhibition in a sequential bireactant system occurs if the binding of the inhibitory substrate prevents the binding of the other substrate to its site and results in a dead-end complex (Dixon & Webb, 1979). However, Chandler and Varandani (1975) also observed substrate inhibition of the PDI-catalyzed reduction of insulin by dithiothreitol, suggesting that the mechanism of inhibition may not involve GSH binding to a peptide binding site but may, instead, involve either a reduction of PDI that decreases the activity or a nonproductive reduction of the PDI–insulin complex. The K_i observed for the inhibition by GSH (4.4 ± 1.6 mM) is significantly lower than the K_m for GSH (17 ± 4 mM), suggesting that inhibition and turnover involve GSH sites of different apparent affinity.

Peptides show competitive inhibition against insulin and noncompetitive inhibition against GSH (Figure 2), consistent with a random or ordered addition of substrates in which the inhibitory peptides bind to the insulin binding site. The rapid reduction of PDI by GSH in the absence of insulin (Gilbert, 1989) and the importance of the reduction of the active-site disulfide of PDI for catalysis suggest that GSH can interact with the enzyme in the absence of the other substrate, consistent with a random order of addition. The apparent K_m and K_i for GSH are very high (17 ± 4 and 4.4 ± 1.6 mM, respectively) as previously reported for the PDI-catalyzed reduction of the disulfide in the peptide CYIQNC (Gilbert, 1989).

The inhibition of PDI activity by peptides is not restricted to the PDI-catalyzed reduction of insulin by GSH. Inhibition is also observed in the more complex case of the PDI-catalyzed oxidative renaturation of reduced, denatured ribonuclease A (Figure 3). The inhibition is competitive against ribonuclease, suggesting that the peptides are recognized by the site(s) on the enzyme involved in the interaction between PDI and its protein substrate.

The competitive inhibition of PDI by peptides implies the existence of a site that is involved in binding peptides and proteins. The peptides shown in Table I span a length of 3–30 residues, contain all 20 amino acids (at least twice except for Met), have net charges that vary between +7 and −6 at pH 7.5, and show hydrophilicities between −1 and +1 (Kyte & Doolittle, 1982). No correlation was observed between the inhibition constants and charge, hydrophobicity, or sequence except for a tendency of cysteine-containing peptides to inhibit at lower concentrations than peptides that do not contain cysteine (Table I).

For non-cysteine-containing peptides, the major factor that correlates with inhibition of PDI appears to be peptide length (Table I, Figure 4). The observed inhibition constants (K_{obs}) increase with decreasing peptide length. The correlation between K_{obs} and peptide length could arise by two mechanisms. The least likely explanation would involve a peptide (protein) binding site which could bind 30 or more amino acids in a sequence-independent manner. The decreasing K_{obs} with increasing length would result from a larger number of PDI–peptide interactions with the longer peptides as the active site becomes more completely occupied by longer and longer peptides. Alternatively, longer peptides could bind to a peptide binding site that accommodates a limited number of residues in a large number of ways. For example, a peptide of 30 residues (L) could bind to a site capable of accommodating

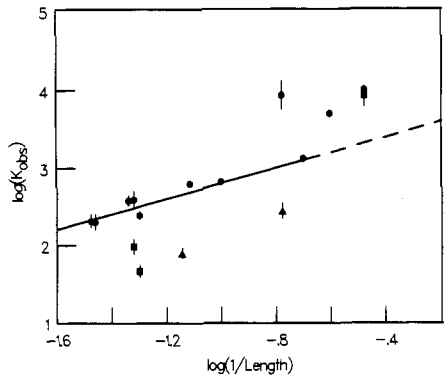

FIGURE 4: Dependence of the observed inhibition constant of the glutathione–insulin transhydrogenase activity of PDI on the chain length of the peptide. Inhibition constants are shown in Table II. (●) Peptides not containing cysteine; (■) peptides containing one cysteine; (▲) peptides containing two cysteines. Error bars are drawn to show the standard deviations of the inhibition constants as determined by nonlinear least squares. Vertical arrows indicate that the plotted value is a lower limit.

5 (m) residues in 34 ($L + m - 1$) different ways, 26 of which would have all 5 binding sites completely occupied, and 8 of which would have incompletely filled sites.

The observed dissociation constant (K_{obs}) resulting from statistical binding of the peptide to PDI will be related to the individual dissociation constants for all possible complexes that could form according to eq 4 where each K represents the

$$1/K_{obs} = 1/K_1 + 1/K_2 + 1/K_3 + ... + 1/K_{L+m-1} \quad (4)$$

dissociation constant of a single complex. For an interaction that is sequence-specific, the dissociation constant corresponding to the specific interaction will be smaller than those for the nonspecific interactions, and one dissociation constant will dominate the expression for K_{obs}. In this case, there would be no reason to expect a relationship between binding and length. On the other hand, if the peptide binding site is indiscriminate with respect to side chain identity, the different microscopic PDI–peptide complexes should exhibit similar dissociation constants.

For peptides significantly longer than the peptide binding site, binding modes in which one or more of the PDI subsites are unoccupied should make a smaller contribution to K_{obs} because they will represent only a small fraction of all the possible binding modes, and the dissociation constant will be larger because some of the binding sites will be unfilled. If species with incompletely filled binding sites are ignored, the observed macroscopic association constant ($1/K_{obs}$) would be proportional to the number of ways in which a peptide could bind (n) times the microscopic association constant for any one of the equivalent complexes with fully occupied sites ($1/K_{int}$):

$$1/K_{obs} = n/K_{int} \quad (5)$$

For peptides that are significantly longer than the number of residues accommodated at the binding site, the number of ways a given peptide can bind (n) will be approximately equal to the peptide length, L ($L \approx L + m - 1$). For long peptides, the sum of eq 4 converts to

$$K_{obs} = K_{int}/L \quad (6)$$

where L is the length of the peptide. According to this model, the lower K_{obs} for the longer peptides results from a larger number of equivalent ways in which the longer peptide can bind to PDI. A plot of log K_{obs} vs log ($1/L$) should be a straight line with slope 1 and an intercept of K_{int}. For the longer peptides that do not contain cysteine, the log of the observed inhibition constant (K_{obs}) appears to be a linear function of the log of the reciprocal peptide length ($1/L$), as predicted by eq 4.

As the peptides become shorter and shorter, the probability increases that binding will leave one or more sites unoccupied, and the relationship of eq 4 should begin to fail. For peptides that are too small to completely fill the binding site, the dissociation constants (K_{obs}) should be significantly greater than that predicted from the relationship established by the longer peptides. Such deviations are noted in Figure 4 for peptides shorter than five residues.

For peptides that do not contain cysteine, the results shown in Figure 4 are generally consistent with this model and suggest that PDI has a binding site for peptide and protein substrates which is designed to bind peptides in a way that does not discriminate between peptides according to amino acid composition. The observation that hexaglycine binds about 8-fold more weakly than expected from its length would suggest that there may be some interaction with side chain groups which are not available in hexaglycine.

Chandler and Varandani (1975) and Varandani et al. (1975) examined the inhibition of the GSH-dependent reduction of insulin by the cysteic acids of insulin chain A and chain B using much lower concentrations of GSH and an assay in which acid-soluble radioactivity resulting from the reduction of labeled insulin was measured, conditions under which the assay coupled to glutathione reductase is too insensitive to detect insulin reduction. Although not noted by Chandler and Varandani (1975), a similar trend of increased inhibition by longer peptides is present in their more limited data.

Cysteine-containing peptides bind approximately 4–8-fold tighter than non-cysteine-containing peptides of the same length. The observation that performic acid oxidation of ERYLKKDQQLLGIWGCSGKLG increases the inhibition constant about 5-fold (Table I) suggests that the cysteine residue contributes significantly to the interaction with PDI. From the somewhat limited number of cysteine-containing sequences examined, it is difficult to determine exactly how the number of cysteine residues, their separation in the sequence, and the length of the peptide contribute to the binding to PDI. If a cysteine residue by itself provided a specific and strong interaction with the binding site, the binding might be expected to be dependent on the number of cysteines and less dependent on length. However, if there is only a slight preference for cysteine, or the requirement for cysteine to occupy a specific position in the sequence, or a preference for a particular spacing of multiple cysteine residues, the observed dissociation constants will not be a simple function of length, cysteine content, or cysteine spacing.

The relatively weak, indiscriminate binding of peptides to PDI is not what might be expected for the usual enzyme with strict substrate specificity. However, PDI must interact with cysteine-containing proteins in which the sequence context around the cysteine residues is quite variable (Thornton, 1981). The lack of specificity, other than an increased preference for cysteine residues, could be attributed to a binding site that recognizes the protein backbone in addition to a specific but somewhat low-affinity binding site(s) for cysteine. A preference for cysteine could facilitate the thiol/disulfide exchange reactions catalyzed by the enzyme if the site allowed juxtaposition of the cysteine residue to the catalytic dithiol/disulfide centers of PDI.

PDI is a dimeric enzyme with four thioredoxin-like domains per dimer. How these domains cooperate in catalyzing protein folding or reduction is not known (Freedman et al., 1988). It

has been suggested that both the GSH and the protein substrate binding sites on PDI are located on these domains which allows for segmental mobility (Freedman et al., 1988) and the flexible accommodation of substrates of various size and multiple disulfide bonds. The enzyme has been shown to catalyze thiol/disulfide exchange with single-chain polypeptides, multichain and multidomain proteins (Hillson et al., 1984; Freedman et al., 1988), and a variety of low molecular weight thiols and disulfides. It catalyzes the refolding of the scrambled, reduced form of ribonuclease, bovine pancreatic trypsin inhibitor, lysozyme, and bovine serum albumin, the assembly of the oligomeric immunoglobulins IgM and IgA, and the formation of native insulin from its A and B chains (Hillson et al., 1984; Freedman et al., 1988; Creighton et al., 1980; Roth & Pierce, 1987; Tang et al., 1988). On the other hand, isomerization of disulfide bonds to produce scrambled, nonnative form(s) of a protein may also be catalyzed by PDI in proteins containing metastable disulfides such as insulin (Hillson et al., 1984). Given the variety of reactions catalyzed, PDI must be capable of interacting with peptides and protein segments of different sequence. It is intuitively pleasing that PDI appears to have a protein/peptide binding site that is not totally sequence-specific.

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