Kinetics and specificity of homogeneous protein disulphide-isomerase in protein disulphide isomerization and in thiol–protein-disulphide oxidoreduction

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1. The protein disulphide-bond isomerization activity of highly active homogeneous protein disulphide-isomerase (measured by re-activation of 'scrambled' ribonuclease) is enhanced by EDTA and by phosphate buffers. 2. As shown for previous less-active preparations, the enzyme has a narrow pH optimum around pH 7.8 and requires the presence of either a dithiol or a thiol. The dithiol dithiothreitol is effective at concentrations 100-fold lower than the monothiols reduced glutathione and cysteamine. The enzyme follows Michaelis–Menten kinetics with respect to these substrates; K_m values are 4, 620 and 380 μM respectively. 3. The enzyme shows apparent inhibition by high concentrations of thiol or dithiol compounds ($>10 \times K_m$), but the effect is mainly on the extent of reaction, not the initial rate. This is interpreted as indicating the formation of significant amounts of reduced ribonuclease in these more reducing conditions. 4. The purified enzyme will also catalyse net reduction of insulin disulphide bonds by reduced glutathione (i.e. it has thiol:protein-disulphide oxidoreductase or glutathione:insulin transhydrogenase activity), but this requires considerably higher concentrations of enzyme and reduced glutathione than does the disulphide-isomerization activity. The K_m for reduced glutathione in this reaction is an order of magnitude greater than that for the disulphide-isomerization activity, and the turnover number is considerably lower than that of other enzymes that can catalyse thiol–disulphide oxidoreduction. 5. Conventional two-substrate steady-state analysis of the thiol:protein-disulphide oxidoreductase activity indicates that it follows a ternary-complex mechanism. 6. The protein disulphide-isomerase and thiol:protein-disulphide oxidoreductase activities co-purify quantitatively through the final stages of purification, implying that a single protein species is responsible for both activities. It is concluded that previous preparations, from various sources, that have been referred to as protein disulphide-isomerase, disulphide-interchange enzyme, thiol:protein-disulphide oxidoreductase or glutathione:insulin transhydrogenase are identical or homologous proteins. 7. The assay, nomenclature and physiological role of this enzyme are discussed.

The preparation in good yield of homogeneous PDI from bovine liver (Lambert & Freedman, 1983) provides the opportunity for a characterization of the kinetic properties of this enzyme, which have previously only been studied in crude or partially purified preparations.

A considerable body of work shows that microsomal fractions and partially purified preparations of

Abbreviations used: PDI, protein disulphide-isomerase; TPOR, thiol:protein-disulphide oxidoreductase; GIT, glutathione:insulin transhydrogenase; GSH and GSSG, reduced and oxidized glutathione respectively.

PDI catalyse disulphide formation and rearrangements leading to formation of native disulphide pairings in a wide range of substrate proteins, including multi-domain and multi-chain proteins (for review see Freedman & Hillson, 1980). Details of the action of PDI as a catalyst of individual steps in the refolding and oxidation of reduced bovine pancreatic trypsin inhibitor have been studied: findings with crude enzyme (Creighton *et al.*, 1980) have been confirmed with highly purified material (Freedman *et al.*, 1981).

The observation that the molecular properties of

homogeneous bovine liver PDI are very similar to those of preparations described as thiol:protein disulphide oxidoreductase (TPOR) or glutathione:insulin transhydrogenase (GIT) and active in the catalysis of insulin reduction by GSH makes it important to test the activity of purified PDI in this reaction. This provides the opportunity to resolve some controversial results. Several authors have purified to homogeneity enzymes catalysing oxidation-reduction between GSH and insulin disulphide bonds and shown that these preparations are able to catalyse re-activation of 'scrambled' ribonuclease (ribonuclease reduced and then re-oxidized in denaturing conditions to give a complex mixture of non-native disulphide bonds), i.e. that these preparations, termed GIT or TPOR, have PDI activity (Anson *et al.*, 1973b; Varandani *et al.*, 1975; Morin *et al.*, 1978; Roth *et al.*, 1979). They have speculated that these two enzyme activities, defined by different assays and EC numbers (GIT and TPOR, EC 1.8.4.2.; PDI, EC 5.3.4.1) are expressions of a single protein, but they have not provided quantitative data on the specific PDI activities of their preparations, nor on the relative yields of this activity and the GIT/TPOR activity; quantitative co-purification of the two activities has not been established.

In the present paper we study the kinetics and thiol-specificity of the homogeneous enzyme in the standard assay reaction, the re-activation of 'scrambled' ribonuclease. We also examine its activity in the net reduction of protein disulphide bonds in insulin. We show that, at high concentrations of GSH and enzyme, purified PDI is active in the reduction of insulin disulphide bonds, i.e. that it has GIT activity. We establish that the activities co-purify quantitatively, and that the specific activity of our preparation in this reaction is comparable with those of previous preparations described as GIT or TPOR. In view of the considerable similarity in molecular properties among all these preparations, we conclude that the preparations that have been purified from various sources and shown to catalyse protein disulphide isomerization and net protein disulphide reduction are identical or homologous proteins.

Materials and methods

Materials

Pure bovine PDI was prepared by the method of Lambert & Freedman (1982, 1983). The final preparations were stored at -20°C as a freeze-dried powder and retained over 75% of their initial activity after 6 months. Samples were as a routine subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to test for any degradation.

Rat liver microsomal fraction was prepared by gel

filtration of a post-mitochondrial supernatant (Hawkins & Freedman, 1979) and sonicated in four 15 s bursts, with 20 s cooling in between, with an MSE 150 W probe sonicator operated at maximum power. Other materials were from the same sources as indicated in previous publications (see Hillson & Freedman, 1980a).

Assays of enzyme activities

PDI activity was assayed by the method of Ibbetson & Freedman (1976), in which the re-activation of 'scrambled' ribonuclease is monitored by a dual-wavelength spectrophotometric assay of ribonuclease activity. The isomerase sample and buffer (usually 50 mM-sodium phosphate, pH 7.5), in a total volume of 0.9 ml, were incubated with 0.01 ml of dithiothreitol (1 mM stock solution, final concentration $10\mu\text{M}$) for 2 min at 30°C . A 0.1 ml portion of 'scrambled' ribonuclease (0.5 mg/ml in 10 mM-acetic acid, final concentration $4\mu\text{M}$) was added, and at times between 0.5 and 15 min after this addition 0.01 ml samples were withdrawn and assayed for ribonuclease activity. Each sample was added to an assay mixture of 3 ml of TKM buffer (25 mM-KCl/5 mM-MgCl₂/50 mM-Tris/HCl buffer, pH 7.5) containing 0.25 mg of RNA in a quartz cuvette equilibrated at 30°C . Ribonuclease activity was monitored by observing the decrease in A_{260} relative to A_{280} by using the dual-wavelength mode of a Perkin-Elmer 356 spectrometer operating at a slit width of 2.5 nm. The rate of RNA hydrolysis, which was constant over approx. 2 min, gave the ribonuclease activity of the sample. However, where the initial rate was much greater than $0.03\Delta A/\text{min}$, the rate was not maintained constant over a 2 min assay; such assays were rejected and the sample was diluted and re-assayed.

A plot of ribonuclease activity versus time of withdrawal was linear over 15 min; the gradient of this plot gave a measure of the PDI activity (as defined by Ibbetson & Freedman, 1976). Only assays in which this plot had a correlation coefficient >0.99 were accepted as valid. Where necessary, samples were pre-diluted in assay buffer so that the gradient of this plot lay between about 0.7×10^{-3} and $3.0 \times 10^{-3}\Delta A/\text{min}$ per min, the range in which the assay was found to be most sensitive. Assays were performed at least in duplicate and were corrected for any rate of non-enzymic re-activation of 'scrambled' ribonuclease, which was usually less than $0.2 \times 10^{-3}\Delta A/\text{min}$ per min. Fig. 1 shows typical assay data; each trace is a ribonuclease assay, and a vertical series such as Fig. 1(b) or 1(c) represents an assay of PDI. For assays with homogeneous PDI, 2–3 μg of protein was used.

TPOR activity was assayed by a modification of the method of Katzen & Tietze (1966), in which the enzyme-catalysed reduction of the disulphide bonds

of insulin by GSH is linked to the reduction of GSSG to GSH, by NADPH and glutathione reductase; the linked reaction is monitored spectrophotometrically at 340 nm.

The assay was performed in 1 ml cuvettes containing finally 8 mM-GSH, 30 μ M-insulin, 120 μ M-NADPH, 1 unit of glutathione reductase, 5 mM-EDTA and enzyme (usually 40 μ g of pure protein) in 200 mM-sodium phosphate buffer, pH 7.5. The assay mixture (without insulin or enzyme) was equilibrated at 30°C for 2 min in a Pye–Unicam SP. 1800 double-beam recording spectrophotometer. After equilibration, the rate of NADPH oxidation was followed against a reference cuvette containing NADPH, EDTA and buffer only. The rate observed at this stage was due to the spontaneous oxidation of GSH. [If this rate was significantly greater than 0.01 ΔA /min, the stock GSH solution (80 mM) was discarded and another was prepared until a low rate of spontaneous oxidation was obtained; uncontrolled variation between stock solutions in the rate of spontaneous oxidation was not overcome by saturating all solutions with N₂, by increasing the EDTA concentration or by other attempted modifications.] Insulin was then added, and the rate of NADPH oxidation was monitored to give a measure of the non-enzymic rate of GSH–insulin oxidoreduction. After a stable non-enzymic rate had been maintained for 2–3 min, pure enzyme was added and, after mixing, the rate of NADPH oxidation due to TPOR enzymic activity was observed. Rates were linear for up to 5 min. All assays were performed at least twice, and enzymic rates were corrected for control rates of NADPH oxidation.

Results

PDI activity

A number of buffers have been used in previous work on PDI. Table 1 gives the relative activities for a preparation of pure enzyme assayed in several such buffer systems. Activities are expressed relative to that in TKM buffer, and it is clear that the addition of sucrose or the omission of the inorganic salts had no significant effect on activity. However, the presence of EDTA or of phosphate buffer significantly enhanced activity by >60% and >30% respectively. The mechanism of this activation was not established, but it may involve chelation of trace heavy-metal ions that otherwise interact with thiol groups. Since the effects of EDTA and phosphate are not additive (Table 1), it is likely that they operate by the same mechanism. Results comparable with the data in Table 1 were also observed with partially purified preparations of the bovine liver enzyme and with sonicated rat liver microsomal fraction (results not shown). Enhancement by

Table 1. PDI activity assayed in different incubation buffers

PDI activity was assayed as described in the Materials and methods section. A 2.5 μ g portion of homogeneous bovine liver PDI was used, with dithiothreitol (10 μ M) and 'scrambled' ribonuclease (50 μ g/ml) as substrates. Activities were related to the value obtained with TKM buffer as incubation buffer. The results are given as means $\pm$ s.d. for the numbers of assays performed indicated in parentheses. Key to buffer components: Tris, 50 mM-Tris/HCl buffer, pH 7.5; K⁺, 25 mM-KCl; Mg²⁺, 5 mM-MgCl₂; EDTA, 5 mM; phosphate, 50 mM-sodium phosphate buffer, pH 7.5; sucrose, 0.25 M.

Incubation buffer, pH 7.5	Relative PDI activity
Tris	1.05 $\pm$ 0.09 (3)
Tris, K ⁺ , Mg ²⁺	1.00 $\pm$ 0.15 (10)
Tris, EDTA	1.67 $\pm$ 0.27 (3)
Phosphate	1.31 $\pm$ 0.20 (10)
Phosphate, K ⁺ , Mg ²⁺	1.47 $\pm$ 0.23 (5)
Phosphate, EDTA	1.61 $\pm$ 0.29 (5)
Sucrose, Tris, K ⁺ , Mg ²⁺	1.10 $\pm$ 0.05 (5)
Sucrose, Tris, EDTA	1.68 $\pm$ 0.31 (5)

EDTA of PDI activity in microsomal fraction has been shown previously, but was interpreted as exposure of the enzyme as a result of EDTA-induced detachment of ribosomes (Williams *et al.*, 1968). This cannot account for the finding now reported that comparable activation is observed with the pure enzyme in solution.

In view of the results, phosphate buffer was selected for most of the kinetic work. Fig. 1(d) shows an examination of the linearity of the basic assay method. In the absence of enzyme the rate of spontaneous re-activation of 'scrambled' ribonuclease in our conditions was close to 0.2 $\times 10^{-3}$ ΔA /min per min (Fig. 1a), and a rate 3-fold greater than this was observed on addition of 0.5 μ g of pure bovine liver PDI (Fig. 1b). This was the smallest amount that could be assayed reliably. PDI activity increased with increasing enzyme added, up to about 3 μ g of pure enzyme (Fig. 1d). With greater amounts the appearance of ribonuclease activity was so rapid that linear rates could not be obtained without altering the basic procedure. It is important to emphasize that, in all our work, PDI assays are a true measure of the rate of increase of ribonuclease activity in that each isomerase assay involved the removal at intervals of several (at least five) samples from an incubation mixture and their immediate assay for ribonuclease activity. Only PDI assays in which the rate of increase of ribonuclease activity was linear with time were accepted. The specific activity of the pure PDI was close to 1000 units/g of protein. By calibrating the assay with authentic fully active ribonuclease it was possible to calculate that the turnover number is of the order of 1–10 min⁻¹. This, of course, does not represent the turnover

Fig. 1. Assay of PDI by re-activation of 'scrambled' ribonuclease

The principles and experimental details are described in the Materials and methods section. (a) Re-activation of 'scrambled' ribonuclease ($4 \mu\text{M}$) by dithiothreitol ($10 \mu\text{M}$) in the absence of PDI. The plot of these data as ribonuclease activity versus incubation time gives a rate of appearance of ribonuclease activity of $0.17 \times 10^{-3} \Delta A/\text{min per min}$ (correlation coefficient 0.999). This is the non-enzymic control rate of re-activation of 'scrambled' ribonuclease. (b) As for (a), but in the presence of $0.5 \mu\text{g}$ of pure PDI. The series of traces shown here correspond to a rate of re-activation of 'scrambled' ribonuclease of $0.72 \times 10^{-3} \Delta A/\text{min per min}$ (correlation coefficient 0.999). (c) As for (b), but in the presence of $2.5 \mu\text{g}$ of pure PDI. The series of traces shown here correspond to a rate of re-activation of 'scrambled' ribonuclease of $2.58 \times 10^{-3} \Delta A/\text{min per min}$ (correlation coefficient 0.999). (d) Results obtained from assays such as those shown in (a)–(c) plotted as rate of re-activation of 'scrambled' ribonuclease versus amount of pure PDI present in the assay mixture.

number in a single specific chemical reaction, but in terms of molecules of 'scrambled' ribonuclease converted into fully active native ribonuclease; the number of individual thiol–disulphide interchange reactions involved in this re-activation process is not known and presumably is not identical for each molecule, since the substrate is heterogeneous in relative molecular mass and in disulphide pairing. It should be stressed that this value refers to the standard assay, in which a patently non-physiological substrate is used.

The kinetic properties of this preparation of bovine PDI are close to those previously observed with less-active and less-pure material (Hawkins & Freedman, 1976; Hillson & Freedman, 1980a). In phosphate buffers the pH optimum was 7.8. The enzyme showed hyperbolic steady-state kinetics with respect to thiol substrates; Table 2 shows Michaelis–Menten parameters for three alternative thiols and for 'scrambled' ribonuclease. The K_m values for the monothiols were two orders of magnitude greater than that for the dithiol dithiothreitol, but there was

no significant difference between those for the two monothiols despite their opposite charge.

At high concentrations of dithiothreitol inhibition was observed, and Fig. 2 shows that this was time-dependent, i.e. that the re-activation of the ribonuclease became increasingly non-linear with increasing dithiothreitol concentration. A single fixed-time-point assay would only show inhibition by high dithiothreitol concentrations, whereas the full assay shows that the initial rate of ribonuclease activation was not affected. The apparent inhibition by dithiothreitol was therefore probably an effect on the yield of native ribonuclease at equilibrium. When the concentration of dithiothreitol in the incubation mixture is more than 4-fold that of 'scrambled' ribonuclease (approx. $16 \mu\text{M}$), there is an excess of dithiol over disulphide in the assay mixture as a whole, and a significant amount of reduced ribonuclease would be expected to form from 'scrambled' ribonuclease. Similar effects were noted when very high ($>6 \text{ mM}$) concentrations of monothiols were used in the assay.

Table 2. *Steady-state kinetic parameters for homogeneous PDI*

Kinetic parameters were obtained by computer analysis of direct linear plots (Eisenthal & Cornish-Bowden, 1974) and by linear-regression analysis of Hanes and Eadie-Hofstee plots. For the thiol substrates and insulin, the values found by these three methods were in good agreement, and the value given is that derived from the direct linear plot. Units of PDI activity are as defined by Ibbetson & Freedman (1976); units of TPOR activity are μmol of GSSG formed/min. Abbreviation: sRNAase, 'scrambled' ribonuclease.

Assay	Test substrate	Co-substrate	K_m (μM)	V_{max} (units/g)
PDI (sRNAase re-activation)	sRNAase	Dithiothreitol (10 μM)	2*	1000*
	Dithiothreitol	sRNAase (4 μM)	3.9	850
	Cysteamine	sRNAase (4 μM)	380	1108
	GSH	sRNAase (4 μM)	620	1004
TPOR (insulin reduction by GSH)	GSH	Insulin (30 μM)	7150	262
	Insulin	GSH (8 mM)	3.1	198

* For 'scrambled' ribonuclease the data did not give a good fit to the Michaelis-Menten equation; the value for V_{max} is derived by inspection and a value for $s_{0.5}$ (not K_m) obtained by interpolation.

Fig. 2. Inhibition of PDI activity by high concentrations of dithiothreitol

The Figure shows the time course of re-activation of 'scrambled' ribonuclease by PDI at various concentrations of dithiothreitol. Full experimental details are given in the Materials and methods section. The plot at 10 μM -dithiothreitol is linear and is a standard determination of PDI activity; those for the higher concentrations of dithiothreitol are increasingly nonlinear: ●, 10 μM ; ■, 30 μM ; ▲, 50 μM ; ▼, 70 μM ; ○, 100 μM ; □, 200 μM . The data are corrected for control rates in absence of PDI at the corresponding dithiothreitol concentration.

The purified enzyme was very sensitive to inhibition by low concentrations of detergent. Both sodium deoxycholate and Triton X-100 at 0.01% (v/v) inhibited activity by 50–60%, but no further inhibition was observed when the detergent concentrations were increased to 0.1% (v/v). Sensitivity of the enzyme to one or other of these detergents has been reported before in rat liver microsomal fraction (Ibbetson & Freedman, 1976; Ohba *et al.*, 1977), in bovine liver microsomal fraction (Freed-

Fig. 3. Variation of TPOR activity with GSH concentration

TPOR activity, the enzymic catalysis of reduction of insulin disulphide bonds by GSH, was assayed by the spectrophotometric linked assay method described in the Materials and methods section. The insulin concentration was 30 μM . Reaction was initiated by addition of 40 μg of the pure enzyme. The data at each GSH concentration are corrected for the control non-enzymic rate observed at that GSH concentration. ●, Enzymic activity; ○, control.

man *et al.*, 1978), in partially purified bovine liver PDI (Hawkins & Freedman, 1976) and in the homogeneous enzyme from rat liver (Ohba *et al.*, 1981), but in general greater inhibition was observed than that noted in the present work. This effect is not due to the presence of traces of Triton X-100 in the purified enzyme used in the present work (see Lambert & Freedman, 1983), since the same results were obtained with enzyme before and

after treatment with Amberlite to remove the detergent. Similar partial inhibition of PDI activity by low concentrations of detergent was noted by Morin *et al.* (1978).

TPOR activity

The assay of enzyme-catalysed reduction of protein disulphides by thiol compounds has conventionally employed insulin as the protein substrate and GSH as the thiol. Of the methods available (Chandler & Varandani, 1975), the linked spectrophotometric assay is clearly to be preferred (see the Discussion section), but it measures NADPH oxidation coupled to the formation of GSSG, making it crucial to minimize other sources of NADPH oxidation and to distinguish the formation of GSSG due to reduction of insulin disulphides from that due to oxygen, or other oxidants, catalysed by trace metal ions or other enzymes. To minimize this spontaneous oxidation, low concentrations of GSH (1–2 mM) have conventionally been used in the linked spectrophotometric assay. At this concentration of GSH, and at enzyme concentrations used for the PDI assay, the pure preparation of PDI had barely detectable TPOR activity, as shown for previous preparations (Hillson & Freedman, 1980a). However, when the GSH and enzyme concentrations were raised, and appropriate correction was made for other sources of GSH oxidation, the preparation was found to catalyse the net reduction of insulin disulphides (Fig. 3). This Figure shows that the 1–2 mM-GSH used as a routine in the past (Ansorge *et al.*, 1973; Chandler & Varandani, 1975; Morin *et al.*, 1978) is well below the K_m for GSH. The highest practicable concentration of GSH for routine assay was 8 mM; above this it became increasingly difficult to eliminate or correct reliably for non-enzymic oxidation.

With 8 mM-GSH as the standard concentration a linked spectrophotometric assay is possible, and, as shown in Fig. 4, the measured activity was linear with the amount of pure PDI assayed. The specific activity was approx. 200 μ mol of GSSG formed/min

per g, corresponding to a turnover number of 12 min^{-1} . At least 20 μ g of pure enzyme was required for the assay of its TPOR activity, as compared with the 2 μ g taken in the standard assay of PDI activity. The dependence of reaction rate on insulin concentration is hyperbolic and gives a K_m of approx. 3 μ M. At the insulin concentration used as a routine in the present work (30 μ M), GSSG formation was linear for 5 min, with no detectable interference from insulin polymerization (Varandani, 1966) within this time. Analysis of the reaction kinetics with variation of both thiol and disulphide

Fig. 4. Variation of TPOR activity with enzyme concentration

The assay was performed as described in the Materials and methods section, except that in this case the reaction was initiated by the addition of insulin rather than enzyme. Data are corrected for the non-enzymic rate; this was in the range 0.007–0.010 $\Delta A/\text{min}$.

Table 3. Co-purification of PDI and GIT activities through a typical preparation of bovine liver enzyme

The enzyme was purified from 500 g of bovine liver by using the procedure of Lambert & Freedman (1982, 1983). Samples of the various stages of the preparation were monitored for PDI and TPOR activity by using the assays described in the text. TPOR activities were not measured in the first two stages because of interference from other activities (see the Discussion section). Values in parentheses denote degree of purification relative to the 55–85%-satn.-(NH_4)₂SO₄ fraction.

Stage	PDI specific activity (units/g)	Purification (fold)	TPOR specific activity (units/g)	Purification (fold)
Homogenate	4	1	—	—
Heat-treatment supernatant	14	3.5	—	—
55–85%-satn.-(NH_4) ₂ SO ₄ fraction	48	12 (1)	14.7	(1)
CM-Sephadex, pH 5.3 eluate	180	45 (3.8)	89.4	(6.1)
DEAE-Sephacel, pH 6.3 eluate	950	238 (19.8)	210	(14.3)

substrates gave a series of Hanes plots intersecting in the third quadrant (results not shown), implying a ternary-complex mechanism.

The observation that a homogeneous preparation of PDI has TPOR activity makes it clear that this is an intrinsic activity, not due to a contaminant. The fact that a single protein is responsible for both these activities is confirmed by the observations that the two activities co-purified quantitatively through the preparation of PDI (Table 3) and that they were equally inhibited by detergents (Morin *et al.*, 1978; N. Lambert & R. B. Freedman, unpublished work).

Discussion

The key issues arising from the present study concern the relationship between the two activities of this enzyme. It has previously been shown that these two processes involve essentially the same basic chemical reaction, namely thiol-disulphide interchange, and that this reaction is involved in a large number of physiological processes, most of them not well characterized (Freedman, 1979). The questions arising from the current work are as follows. (1) What is the relationship between the various preparations that have been isolated, and shown to catalyse protein disulphide isomerization and/or thiol-protein-disulphide oxidoreduction? (2) What are the physiological functions of these enzymes?

Relationship between the various activities

The observation that a homogeneous preparations of PDI catalyses reduction of insulin disulphide groups by GSH and that this activity co-purifies quantitatively with PDI activity through the final stages of purification establishes that the two activities reside in the same protein species. It is important to note that the standard concentration of GSH in the linked spectrophotometric assay used in this work was 8 mM. Previous work in our laboratory (Hawkins & Freedman, 1976; Ibbetson & Freedman, 1976; Hillson & Freedman, 1980*a,b*), following procedures developed and used by other workers in the field (Chandler & Varandani, 1975; Morin *et al.*, 1978), employed 1–2 mM-GSH. Since this is well below the K_m , the results obtained in all previous work with this assay must be regarded as suspect. With the higher concentration of GSH, a specific activity for our enzyme of 220 μmol of GSSG formed/min per g of protein is obtained. It is difficult to make comparisons with the specific activities reported for previous preparations because of differences in assay conditions, source material etc. However, previous values reported (based on the spectrophotometric linked assay method) are 41 μmol /min per g for bovine pancreatic GIT (Chander & Varandani, 1975), 113 μmol /min per g for bovine liver TPOR (J. E. Dixon, personal

communication) and 183 μmol /min per g for rat liver GIT (Ansorge *et al.*, 1973). The first two values were obtained with assays with a GSH concentration of 1 mM, and the last with a concentration of 4.5 mM, supporting the conclusion that the enzyme is not saturated with GSH at the lower concentrations. With this reservation, it appears that our enzyme preparation is comparable with those previously described in its TPOR activity.

Thus the kinetic data imply that preparations described as PDI, GIT or TPOR are equivalent. The bulk of the structural and molecular data reviewed in the preceding paper (Lambert & Freedman, 1983) also support this conclusion. Therefore we conclude that the preparations derived from various sources, assayed by various reactions and defined by the various names mentioned are identical or homologous proteins.

One can then ask what the most appropriate assay for this protein is. In many contexts immunological determination of the enzyme protein is most suitable, and this approach has been adopted by some authors (Ohba *et al.*, 1981; Roth & Koshland, 1981). For enzymic assay the choice is between the various possible assays based on GSH reduction of insulin disulphide groups, or the assay of disulphide isomerization by re-activation of 'scrambled' ribonuclease. The assay based on loss of insulin immunoreactivity does not follow any specific chemical reaction. That based on release of trichloroacetic acid-soluble fragments from ^{125}I -labelled insulin is claimed to depend on formation of the soluble A-chain after reduction of the interchain disulphide groups. But it is not free of problems; any contaminating proteinase, particularly 'insulinase' (Duckworth *et al.*, 1979), could generate soluble radioactive material as an artifact, and it appears that the extent of precipitation of radioactive peptides is dependent on the nature and concentration of other proteins present in the assay mixture as carriers (Izzo *et al.*, 1972; Varandani, 1973). These two assays are not sensitive to reduction of all three disulphide bonds in insulin (Chandler & Varandani, 1975), and are discontinuous assays, making them unsuitable for many purposes.

The linked spectrophotometric assay does not have these disadvantages, but it is inconvenient in many ways. Firstly, other processes that can lead to net formation of GSSG or oxidation of NADPH must be prevented or corrected for, which is difficult at high GSH concentrations (>8 mM) or in crude preparations, which usually contain NADPH or GSH oxidase activities (see, e.g., Ibbetson & Freedman, 1976). Secondly, because of the enzyme's low turnover number, the spectrophotometric assay is insufficiently sensitive, and 20–30 μg of homogeneous enzyme is required to obtain a convenient

rate of reaction. A final problem for all the assays based on GSH-insulin oxidoreduction is that this is not a specific test for the enzyme species known as GIT or TPOR, since a number of other, clearly distinct, protein species can catalyse this reaction, in particular the small cytosolic proteins thioltransferase (Axelsson & Mannervik, 1980), glutaredoxin (Luthman & Holmgren, 1982) and thioredoxin (in the presence of thioredoxin reductase) (Holmgren, 1979). The assay of protein disulphide isomerization based on re-activation of 'scrambled' ribonuclease is not a continuous assay, and the procedure that we use, involving several assays of ribonuclease activity over a period of 15 min, is labour-intensive. However, the assay is specific and sensitive; the background non-enzymic rate is reasonably small, there is little interference by other enzymic or non-enzymic processes, so that the assay can be used equally well with crude and pure materials, and the substrates can be used at concentrations above their respective K_m values. On balance, this appears to be the most appropriate assay for monitoring the enzyme activity of this protein.

The most suitable name for this enzyme is also debatable. Glutathione:insulin transhydrogenase is clearly unsuitable, since the enzyme is not specific for either GSH or insulin (although these substrates are commonly used in assays) and the reaction catalysed is not strictly a transhydrogenation (Askelof *et al.*, 1974). The name thiol:protein-disulphide oxidoreductase accurately describes one expression of the enzyme's activity, but, as mentioned above, other proteins also display this activity. Protein disulphide-isomerase is distinctive but more limited, and describes the overall effect of the enzyme in some conditions rather than the precise chemical reaction catalysed (see Creighton *et al.*, 1980). Protein-disulphide interchange enzyme may be more appropriate, but is not recognized by the Enzyme Commission. Since no terse and unambiguous name can conveniently summarize the various enzymic activities of this species, a final decision should probably be based on consideration of the enzyme's physiological role.

Physiological role of the protein

The physiological role of the enzyme is likely to be associated with protein disulphide bonds, since it is a comparatively poor catalyst of oxidoreduction and interchange between low- M_r thiols and disulphides (Creighton *et al.*, 1980). Early work on the TPOR activity was conducted in the context of studies on insulin catabolism, and Varandani (1977) has summarized the evidence that the enzyme is involved in this process. However, many properties of the enzyme argue against such a role. The enzyme shows no specificity for insulin disulphide bonds,

and its K_m for insulin is several orders of magnitude greater than physiological insulin concentrations. By contrast, a GSH-independent insulin-specific proteinase has been described whose K_m for insulin is in the range 0.01–0.1 μM (Goldstein & Livingston, 1981). Compared with other species that can catalyse reduction of insulin disulphide groups, the protein known as GIT or TPOR is actually a very poor catalyst; its turnover number in this reaction of 12 min^{-1} is 500-fold lower than that of the cytosolic thioltransferase (Axelsson *et al.*, 1978) in the same reaction. There is no correlation between the tissue distribution of the enzyme and that of insulin catabolism, and the enzyme is located almost entirely in the endoplasmic reticulum, whereas recent evidence suggests that insulin degradation proceeds by a receptor-mediated endocytotic pathway with the internalized insulin ultimately being degraded in lysosomes (Plas & Desbuquois, 1982; Krupp & Lane, 1982). GIT activity is also observed in preparations of PDI from sources such as wheat germ and endosperm (L. T. Roden & R. B. Freedman, unpublished work), where insulin catabolism could not possibly be the enzyme's physiological role. These findings taken together suggest that the enzyme is not involved in catabolism of protein disulphide bonds, and certainly does not function specifically in insulin catabolism.

Observation of the enzyme's disulphide-interchange activity and its catalysis of formation of native disulphide bonds from reduced or 'scrambled' proteins led to the suggestion that the enzyme acts *in vivo* to ensure rapid formation of native disulphide bonds (Epstein *et al.*, 1963). Recent evidence in favour of this proposal has been summarized by Freedman *et al.* (1983). Essentially the evidence is based on the enzyme's (1) distribution, (2) developmental properties, (3) subcellular location and (4) specificity. (1) The enzyme is widely distributed and is mostly concentrated in tissues actively synthesizing disulphide-bonded proteins, such as liver, pancreas, tendon and spleen. In lymphoid cells the enzyme is 10–100-fold more active in cells secreting immunoglobulins than in non-secreting cells (Roth & Koshland, 1981). In the developing chick embryo, in which procollagen is the major disulphide-bonded protein synthesized, the enzyme's tissue distribution matches that of procollagen biosynthesis and those of enzymes that specifically modify procollagen (Brockway *et al.*, 1980). (2) In chick-embryo tissues the enzyme activity varies through development in parallel with variations in the specific rate of procollagen synthesis and in parallel with changes in enzymes that specifically modify procollagen (Brockway *et al.*, 1980). In wheat, the enzyme is found in the developing endosperm, where storage proteins are deposited, and is most active during the mid and late phases of endosperm development,

when the specific rate of synthesis of disulphide-bonded storage proteins is maximal (Roden *et al.*, 1982; Freedman *et al.*, 1983). (3) In all sources studied, including developing wheat endosperm, the enzyme is located in the endoplasmic reticulum, the site of synthesis, translocation and post-translational modification of disulphide-bonded secretory proteins. (4) The enzyme has a broad protein specificity and is active on multi-chain and multi-domain proteins as well as the small proteins commonly used in assays (see, e.g., Freedman & Hillson, 1980).

In addition to these lines of evidence, the fact that the enzyme is highly abundant (0.4% of liver protein, over 2% of liver microsomal protein) and the preliminary evidence for conservation of structure (Freedman *et al.*, 1983) imply that the enzyme performs a significant cellular function.

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