A direct, continuous, sensitive assay for protein disulphide-isomerase based on fluorescence self-quenching

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PDI (protein disulphide-isomerase) activity is generally monitored by insulin turbidity assay or scrambled RNase assay, both of which are performed by UV–visible spectroscopy. In this paper, we present a sensitive fluorimetric assay for continuous determination of disulphide reduction activity of PDI. This assay utilizes the pseudo-substrate diabz-GSSG [where diabz stands for di-(*o*-aminobenzoyl)], which is formed by the reaction of isatoic anhydride with the two free N-terminal amino groups of GSSG. The proximity of two benzoyl groups leads to quenching of the diabz-GSSG fluorescence by approx. 50% in comparison with its non-disulphide-linked form, abz-GSH (where abz stands for *o*-aminobenzoyl). Therefore the PDI-dependent disulphide reduction can be monitored by the increase in fluorescence accompanying the loss of proximity-quenching upon conversion of diabz-

GSSG into abz-GSH. The apparent K_m of PDI for diabz-GSSG was estimated to be approx. 15 μ M. Unlike the insulin turbidity assay and scrambled RNase assay, the diabz-GSSG-based assay was shown to be effective in determining a single turnover of enzyme in the absence of reducing agents with no appreciable blank rates. The assay is simple to perform and very sensitive, with an estimated detection limit of approx. 2.5 nM PDI, enabling its use for the determination of platelet surface PDI activity in crude sample preparations.

Key words: denitrosation, diabz-GSSG, disulphide exchange, fluorescence self-quenching, insulin turbidity, protein disulphide-isomerase.

INTRODUCTION

PDI (protein disulphide-isomerase) is an approx. 110 kDa homodimeric protein widely distributed across eukaryotic tissues, making up approx. 1% of the total protein content of cells [1]. One of the most studied functions of PDI is its ability to catalyse isomerization and rearrangement of disulphide bonds in the endoplasmic reticulum [2]. Recently, its S-denitrosation activity has also been demonstrated [3] and characterized [4].

To date, the two most widely used methods for determining PDI activity are insulin turbidity assay and scRNase (scrambled RNase) assay. Insulin turbidity assay is based on monitoring the increase in turbidity at 630 nm caused by the reduction of disulphide bond between the α - and β -chains of insulin [5]. scRNase assay is based on PDI-dependent isomerization of scRNase to its catalytically active native form that, in turn, acts on its substrate RNA [6] or cCMP [7], resulting in the increase in absorbance monitored at 260 or 295 nm respectively. These assays are performed in the presence of reducing agents like DTT (dithiothreitol) or GSH that are required to reduce the enzyme active site after one turnover. Although the assays are widely used for estimating enzyme activity, there are several issues associated with them: (i) there is always a significant non-enzymatic blank rate due to the presence of reducing agent that makes the assays unsuitable for detecting activity in samples with smaller enzyme concentrations; (ii) long lag phases make it difficult to estimate true initial rates; (iii) non-stoichiometric increase in the enzymatic rates with insulin assay with stoichiometric increase in enzyme concentration; (iv) the assays are insensitive to study a single turnover of enzyme in the absence of reducing agent or at lower concentrations of reducing agents; and (v) the assays cannot be performed with precision in crude samples like platelet suspensions containing small amounts of the enzyme.

When two identical fluorescent molecules are in close proximity, their fluorescence is quenched due to intermolecular interactions. This phenomenon is termed FSQ (fluorescence self-quenching). Fluorescence probes undergoing FSQ have been exploited in the past for estimating protease activity [8], protein dimerization [9] and protein folding [10]. We have previously described that abz-S-nitrosohomocysteine (where abz is *o*-aminobenzoyl) could be used as a probe for thiol detection [11]. The excitation overlap between IA (isatoic anhydride) and S-NO (the bond between S and NO in S-nitrosothiols; 343 nm) quenches the fluorescence of the former, which, in turn, could be removed by denitrosating the probe with the help of thiols. Fluorescence-quenched peptides have been used in the past for quantitative analysis of PDI disulphide reduction activity using disulphide-linked synthetic peptides containing a fluorescent probe and a quencher [12,13]. The PDI pseudo-substrate presented here is prepared by a single-step synthesis using readily available GSSG. Furthermore, the observed self-quenching of the fluorescent probes in the disulphide-linked probe negates the need for incorporation of quencher. The assay was optimized to monitor disulphide reduction activity with purified human recombinant PDI and platelet surface PDI in a standard fluorimeter and shown to be more kinetic-friendly and more sensitive compared with commonly used PDI assays.

MATERIALS AND METHODS

Materials

GSH and GSSG, PAO (phenylarsine oxide), DTT, EDTA, Cu(II) chloride and Sephadex G-10 were purchased from Sigma–Aldrich (Oakville, ON, Canada). The monoclonal anti-PDI antibody

Abbreviations used: abz, *o*-aminobenzoyl; PDI, protein disulphide-isomerase; csPDI, cell-surface PDI; diabz, di(*o*-aminobenzoyl); DTT, dithiothreitol; FSQ, fluorescence self-quenching; IA, isatoic anhydride; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MS/MS, tandem MS; PAO, phenylarsine oxide; PSD, post-source decay; scRNase, scrambled RNase.

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RL90 was purchased from Abcam (Cambridge, MA, U.S.A.). The Bradford reagent was obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

PDI assay buffer

PDI assay buffer contained 0.1 M potassium phosphate buffer (pH 7.0) and 2 mM EDTA. This buffer was used throughout the study unless otherwise specified.

Preparation of diabz-GSSG [di-(*o*-aminobenzoyl)-GSSG]

GSSG was incubated with 10-fold molar excess of IA in phosphate buffer (100 mM sodium phosphate and 2 mM EDTA, pH 8.5) for 4 h at room temperature (25 °C). This sample (100 μ M) was then passed through a Sephadex G-10 column (100 mm $\times$ 10 mm) and 500 μ l aliquots were collected with the help of a fraction collector. The samples were tested for maximum fluorescence on a Varian Cary Eclipse fluorescence spectrometer with excitation at 312 nm and emission at 415 nm after the addition of 10 mM DTT. All the fractions that showed 95–100 % increase in the fluorescence were pooled and stored at -80°C .

Preparation of abz-GSH

abz-GSH was prepared by treating 10 mM diabz-GSSG with 100 mM DTT for 1 h and then separating the mixture with a Sephadex G-10 column. The first few aliquots showing high absorbance A at 312 nm were pooled together and quantified using molar absorption coefficient $\epsilon = 4600 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Autoxidation of abz-GSH (10 μ M) was studied in the presence of Cu^{2+} (10 μ M).

Quantification of [abz-GSH] formation

Increase in fluorescence was monitored as a function of [abz-GSH] ($\epsilon = 4600 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and the standard plot was generated with excitation at 312 nm and emission at 415 nm. This standard plot was used wherever quantification of the reduction of [diabz-GSSG] to [abz-GSH] was required.

MS

GSSG and the purified diabz-GSSG were analysed by MALDI-TOF-MS (matrix-assisted laser-desorption ionization-time-of-flight MS). All spectra were acquired on a Voyager DE-Pro (Applied Biosystems, Indianapolis, IN, U.S.A.) equipped with a nitrogen laser operating at 337 nm. The matrix used was cyanocinnamic acid dissolved in 50 % acetonitrile containing 1 % formic acid. MALDI-PSD (where PSD stands for post-source decay) was performed to obtain structural information on the parent ions.

PDI purification

Recombinant human PDI was expressed using the *Escherichia coli* strain BL21 (DE3) and expression vector pET-28a. This plasmid encodes a fusion protein containing the entire human PDI sequence with an N-terminal His₆ tag [14]. Recombinant PDI was purified from the soluble fraction of the cell lysate using Ni-CAMTM HC resin (Sigma), which is a high-capacity nickel-affinity matrix. Bound PDI was eluted using 250 mM imidazole in 50 mM Tris/HCl (pH 8.0) and collected in 2.0 ml fractions. The fractions containing PDI were pooled and dialysed against 0.1 M potassium phosphate buffer (pH 7.0). Protein quantification was performed by the Bradford assay [15].

Kinetics of PDI-dependent disulphide reduction

PDI disulphide reduction activity was monitored in PDI assay buffer by adding PDI (2.5 nm–1 μ M) to diabz-GSSG (15 μ M) in the presence of 30 μ M DTT. The increase in fluorescence was monitored at 415 nm with excitation at 312 nm. The activity was inhibited using 10 μ M PAO [16].

Cu^{2+} -catalysed oxidation of abz-GSH

Cu^{2+} (10 μ M) was added to the cuvette containing abz-GSH ($\sim 10 \mu$ M) in 100 mM phosphate buffer, and the fluorescence change was monitored with excitation at 312 nm and emission at 415 nm. After 5 min, 50 μ M DTT was added, followed by the addition of 0.5 μ M PDI to the sample, and fluorescence change was monitored for another 20 min.

Reduction or oxidation of PDI

PDI (25 μ M) was reduced or oxidized by incubating it with 10 mM DTT or 10 mM GSSG respectively for 3 h at room temperature. The excess of DTT or GSSG was removed by using a Sephadex G-25 column. The thiol content of reduced or oxidized PDI was calculated using the DTNB [5,5'-dithiobis-(2-nitrobenzoic acid); Ellman's reagent] assay [17].

Platelet isolation

Suspensions of washed human platelets were obtained by the method of Mustard et al. [18]. Briefly, samples of peripheral venous blood were mixed 6:1 with acid citrate dextrose (25 g/l trisodium citrate dihydrate, 15 g/l citric acid monohydrate and 20 g/l dextrose). Whole blood was centrifuged (190 g for 15 min at 37°C) to yield platelet-rich plasma. Platelets were isolated by centrifugation (2000 g for 15 min at 37°C) and washed three times in Tyrode-albumin solution (pH 7.4). The first wash contained heparin (2 units/ml) and apyrase (1 unit/ml), the second only apyrase (1 unit/ml) and the third wash contained Tyrode's solution without apyrase and heparin. Platelets were quantified using a haemocytometer.

Monitoring platelet csPDI (cell-surface PDI) activity

Samples of washed human platelets were prepared in Tyrode's solution to a final concentration of $10 \times 10^8 \text{ ml}^{-1}$. diabz-GSSG (15 μ M) was incubated with 10 μ M DTT and platelets (50 μ l), and the activity was monitored continuously as a function of time. In addition, variable volumes of platelets (0, 25 and 50 μ l) were incubated with diabz-GSSG (15 μ M) in 1.2 ml of PDI assay buffer for 15 min and then centrifuged at 2000 g for 5 min. The supernatant was added to a cuvette and fluorescence was monitored with excitation at 312 nm and emission at 415 nm. In addition, 50 μ l of the platelets was incubated with anti-PDI antibodies (10 μ g/ml) in parallel to inhibit the activity of PDI [19,20].

RESULTS

Synthesis of diabz-GSSG

IA is a fluorescent molecule with an anhydride functionality that reacts with terminal amino groups of proteins and peptides [21]. In the present study, IA reacted with GSSG to yield diabz-GSSG (Figure 1).

To confirm successful formation of diabz-GSSG, MS/MS (tandem MS) sequencing was performed (Figure 2). The molecular ion at m/z 851.06 representing diabz-GSSG was detected

Figure 1 Reaction of GSSG with IA

Two molecules of IA react with two free amino groups of GSSG at pH 8.5 to give one molecule of diabz-GSSG.

Figure 2 MALDI-PSD analysis of diabz-GSSG

The Figure shows the MS/MS fragmentation patterns of the diabz-GSSG parent ion (MH⁺) at *m/z* 851 (upper spectrum) and the GSSG standard parent ion (MH⁺) at *m/z* 612.7 (lower spectrum). Fragment ion masses corresponding to structural features of diabz-GSSG and GSSG are labelled. MH⁺-abz, -E and -G indicate the loss of aminobenzoyl, glutamate or glycine respectively. Common fragment ions observed between the standard and diabz-GSSG are indicated by asterisks (upper spectrum).

and subjected to MALDI-PSD sequencing (Figure 2, upper panel). The resulting fragmentation pattern was compared with the fragmentation pattern of a GSSG standard (Figure 2, lower panel) where several diagnostic GSSG fragmentation ions were observed. Additional fragment ions representing the abz moiety were detected to unambiguously verify successful diabz-GSSG synthesis. In particular, the abundant fragment ion mass at *m/z* 603 (Figure 2, upper panel) corresponds to the loss of glutamate-abz. This indicates that the abz moiety is located on the glutamate portion of GSSG as intended.

Fluorescence of diabz-GSSG is sensitive to the reduction of the disulphide bond

diabz-GSSG was fluorescent ($\lambda_{\text{max}} = 312$ nm for excitation and $\lambda_{\text{max}} = 415$ nm for emission); however, upon addition of the thiol reducing agent (250-fold molar excess), the fluorescence increased by approx. 100% (Figure 3). The fluorescent fraction, assumed to be abz-GSH, was separated from the excess DTT by

Figure 3 Fluorescence spectra of diabz-GSSG and abz-GSH

diabz-GSSG (15 μ M) spectrum in PDI assay buffer was taken with excitation at 312 nm and emission at 415 nm (dotted line). The same sample was then incubated with 10 mM DTT for 15 min to completely convert diabz-GSSG into abz-GSH and the spectrum was taken under the same conditions (thick black line). RFU, relative fluorescent units.

chromatography on Sephadex G-10. The fluorescence of this compound was not altered by the addition of 300-fold molar excess of DTT, indicating that the observed enhancement of diabz-GSSG fluorescence upon DTT addition is not due to interactions between DTT and the fluorophore (results not shown). The most likely explanation of the fluorescence enhancement phenomenon is that, in diabz-GSSG, the random movement of the abz residues brings them in close proximity of one another, thus resulting in FSQ. Upon disulphide reduction, the distance constraints are removed and the fluorescence increases. In order to test this hypothesis, molecular dynamic simulations (Alchemy 2000; Tripos, St. Louis, MO, U.S.A.) were performed on diabz-GSSG. The simulations indicated that the aminobenzoyl residues can potentially come as close as 73 nm in a periodic fashion, thus supporting the FSQ hypothesis (Figure 4). Further evidence for the FSQ being related to disulphide-bond formation was obtained from Cu²⁺-catalysed oxidation of abz-GSH. When Cu²⁺ was added to the cuvette containing abz-GSH (~ 15 μ M), a rapid decrease in the fluorescence was observed, suggesting that once the fluorophores are linked via a disulphide bridge the fluorescence is quenched (Figure 5, solid line). When PDI (~ 0.5 μ M) was added to this solution, the fluorescence increased to the same level observed for abz-GSH (Figure 5, dotted line). The results obtained with PDI were very significant in that approx. 10 mM DTT is required to achieve the same thiol reduction rates observed with 0.5 μ M PDI (i.e. the reduction conditions in Figure 3). This suggested that diabz-GSSG could be employed to assay the disulphide reduction activity of PDI as well as other enzymes.

Figure 4 MM3 molecular dynamics simulation of diabz-GSSG performed with Alchemy 2000 (Tripos)

(A) A plot of inter-benzylamino distance as a function of time. The initial temperature was 298 K and the simulation was run for 4 ps. (B) The predicted diabz-GSSG conformation at a minimum inter-benzylamino distance (circles). 1 Å = 0.1 nm.

Figure 5 Cu^{2+} -catalysed oxidation of abz-GSH

Cu^{2+} (10 μM) was added to the cuvette containing abz-GSH ($\sim 15 \mu\text{M}$) in phosphate buffer (100 mM, pH 7) and the fluorescence decrease was monitored at room temperature with excitation at 312 nm and emission at 415 nm (solid line). When PDI ($\sim 0.5 \mu\text{M}$) was added to this solution after 5 min, the fluorescence increased to the same level as observed for abz-GSH (solid line). No change in the fluorescence was observed in the sample containing no Cu^{2+} (dotted line). The total fluorescence produced was converted into [abz-GSH] via a standard curve.

Figure 6 Detection of enzymatic activity and its inhibition by PAO

diabz-GSSG (15 μM) was incubated with DTT (50 μM) in PDI assay buffer (pH 7) at room temperature in the presence of 0.5 μM PDI (Δ), 0.5 μM PDI blocked by PAO ($\square$) or DTT alone ($\diamond$). RFU, relative fluorescent units.

Figure 7 Linearity of enzymatic activity

diabz-GSSG (15 μM) was incubated with DTT (50 μM) in PDI assay buffer (pH 7) at room temperature in the presence of various concentrations of PDI (2.5 nM–1 μM), and rates of abz-GSH formation were monitored as a function of time. The total fluorescence produced was converted into the [abz-GSH] formed per minute via a standard curve.

diabz-GSSG is a pseudo-substrate for PDI

diabz-GSSG (15 μM) is resistant to reduction by DTT (50 μM) (Figure 6, $\diamond$). Upon introduction of PDI (0.5 μM) to this solution, the fluorescence increased in a time-dependent manner, thus reporting disulphide bond cleavage (Figure 6, Δ). The PDI activity was completely inhibited in the presence of PAO, a known vicinal thiol blocker [16] (Figure 6, $\square$).

If the fluorescence increase is enzymatic, the initial rates should vary directly with [PDI]. This was shown to be the case (Figure 7) and the assay is sensitive to estimate as low as 2.5 nM PDI activity under the present experimental conditions. The initial rates of abz-GSH formation were monitored as a function of [diabz-GSSG] with a view of estimating the affinity of PDI for diabz-GSSG. The initial rate versus [diabz-GSSG] data were well accommodated by the Michaelis–Menten equation, with an estimated K_m of $15 \pm 1 \mu\text{M}$ (Figure 8).

It is well established that free thiols like DTT [6,20,22,23] or GSH [4,7,24] are required to maintain the thiol–disulphide exchange activity of PDI. Here, PDI (1 μM ; 4 μM in active sites) was incubated with diabz-GSSG (10 μM) and an excess of DTT (50 μM). This yielded approx. 20 μM abz-GSH (Figure 9A, $\square$), whereas diabz-GSSG (10 μM) incubated with DTT (50 μM) alone yielded no fluorescence increase (Figure 9A, $\diamond$). In order to test the ability of diabz-GSSG to detect a single enzyme turnover, PDI (1 μM) was again incubated with diabz-GSSG (10 μM), but only in the presence of enough DTT to support one turnover (i.e. 4 μM) (Figure 9A, Δ). Under these conditions, fluorescence increase equal to approx. 6 μM abz-GSH was generated. This

Figure 8 Estimation of K_m

PDI ($0.5 \mu\text{M}$) was incubated with variable concentrations of diabz-GSSG ($1\text{--}150 \mu\text{M}$) in PDI assay buffer at room temperature, and initial rates of abz-GSH formation were monitored as a function of [diabz-GSSG]. The total fluorescence produced was converted into [abz-GSH] via a standard curve.

Figure 9 A single turnover of enzyme

(A) PDI ($1 \mu\text{M}$) was incubated with diabz-GSSG ($10 \mu\text{M}$) and the disulphide reduction activity was monitored after the addition of $50 \mu\text{M}$ DTT ($\square$). In a separate sample, PDI ($1 \mu\text{M}$) was incubated with diabz-GSSG ($10 \mu\text{M}$) and three separate aliquots of $4 \mu\text{M}$ DTT were added as indicated by arrows ($\triangle$). Fluorescence change of diabz-GSSG ($10 \mu\text{M}$) was also monitored in the presence of $50 \mu\text{M}$ DTT alone ($\diamond$). (B) Completely reduced PDI ($1 \mu\text{M}$), oxidized PDI ($1 \mu\text{M}$) alone or oxidized PDI ($1 \mu\text{M}$) with DTT ($4 \mu\text{M}$) was incubated with diabz-GSSG ($10 \mu\text{M}$), and the abz-GSH formation was monitored for 5 min.

could be repeated with two subsequent additions of DTT ($4 \mu\text{M}$) to the sample. The total fluorescence increase upon addition of three aliquots of DTT corresponded to approx. $16 \mu\text{M}$ abz-GSH, probably as a result of incomplete reduction of enzyme active sites under near-stoichiometric DTT/enzyme ratios.

To illustrate further the sensitivity of the assay, completely reduced PDI ($1 \mu\text{M}$), oxidized PDI alone or oxidized PDI ($1 \mu\text{M}$) with $4 \mu\text{M}$ DTT was incubated with diabz-GSSG ($15 \mu\text{M}$) and the rate was kinetically monitored for 30 min (Figure 9B). While no increase in fluorescence was seen with oxidized PDI, an equivalent

Figure 10 Monitoring platelet csPDI activity

(A) diabz-GSSG ($15 \mu\text{M}$) was incubated with $10 \mu\text{M}$ DTT and the activity was monitored in the absence ($\square$) or presence ($\diamond$) of $50 \mu\text{l}$ of platelets. (B) Platelets ($0, 25$ and $50 \mu\text{l}$) were incubated with $15 \mu\text{M}$ diabz-GSSG in the presence of $10 \mu\text{M}$ DTT for 15 min at room temperature and then centrifuged at $2000 g$. The supernatant was tested for fluorescence at 415 nm with excitation at 312 nm . csPDI activity of platelets ($50 \mu\text{l}$) was completely inhibited in the presence of anti-PDI antibodies ($10 \mu\text{g/ml}$).

fluorescence increase was observed with reduced PDI or oxidized PDI with equimolar DTT (Figure 9B).

Monitoring platelet csPDI activity

Platelets have been shown to have PDI on their surface and its activity has been studied in the past using scRNase assay [25]. However, this discontinuous assay shows significant non-enzymatic rates [25]. In the present study, we have developed a diabz-GSSG assay for monitoring platelet csPDI activity. diabz-GSSG ($15 \mu\text{M}$) was incubated with $10 \mu\text{M}$ DTT and platelets ($50 \mu\text{l}$), and the activity was monitored as a function of time. While no significant increase in fluorescence was observed without platelets (Figure 10A, $\square$), a time-dependent increase was observed in the sample containing platelets (Figure 10A, $\diamond$). This result clearly demonstrates that csPDI activity can be continuously monitored in a single step using this probe. The sensitivity of the assay can further be improved by centrifuging the samples before reading the fluorescence. To this end, platelets ($0\text{--}50 \mu\text{l}$) were incubated with $15 \mu\text{M}$ diabz-GSSG in the presence of $10 \mu\text{M}$ DTT for 15 min at room temperature and then centrifuged at $2000 g$. The supernatant was tested for fluorescence at 415 nm with excitation at 312 nm . While no increase in fluorescence was observed in diabz-GSSG samples without platelets, stoichiometric increase in fluorescence was observed with a corresponding increase in platelet concentration, suggesting reduction of diabz-GSSG by platelet csPDI (Figure 10B). The activity was completely inhibited by PDI antibodies (Figure 10B).

DISCUSSION

FSQ has been used previously in various biochemical techniques [9,10,26,27], including monitoring enzymatic activity [8,28]. In

the present study, we have used this phenomenon to develop a probe, diabz-GSSG, which has two aminobenzoyl moieties attached to two free amino groups of GSSG. The structure of diabz-GSSG was confirmed by MS. The fragmentation pattern of the diabz-GSSG along with the fragmentation pattern of the authentic GSSG standard revealed diagnostic GSSG fragment ions. Most importantly, fragment ions were detected that corresponded to the benzoyl moieties attached to the glutamate portion of GSSG, unambiguously verifying the successful synthesis of diabz-GSSG.

In order for FSQ to occur, the two aminobenzoyl rings of diabz-GSSG must on average come closer than 100 nm. In order to test this, molecular dynamics simulations were performed. The simulations indicated that the abz residues could potentially come as close as 73 nm in a periodic fashion (Figure 4), suggesting the possibility of FSQ. This hypothesis was confirmed when an approx. 2-fold increase in fluorescence was observed upon complete reduction of the disulphide bond by DTT (10 mM) that removed the distance constraint resulting in the increase of fluorescence (Figure 4). The probe was then tested for enzymatic reduction by PDI in the presence of minimal concentration of DTT (50 μ M). While no increase in fluorescence was observed with DTT alone (Figure 6, $\diamond$), a rapid increase in fluorescence was observed in a time-dependent manner in the presence of PDI (0.5 μ M), suggesting enzymatic reduction of disulphide bond (Figure 6, $\triangle$). The final product of disulphide reduction of one molecule of diabz-GSSG would be two molecules of abz-GSH. If diabz-GSSG were to show the phenomenon of FSQ, there should be approx. 50% decrease in fluorescence upon re-oxidation of abz-GSH to diabz-GSSG. To this end, the autoxidation of abz-GSH was studied in the presence of cupric chloride, a commonly employed thiol-oxidizing agent. The fluorescence decreased rapidly upon addition of cupric chloride to the sample containing abz-GSH, confirming the FSQ due to the oxidation of abz-GSH to diabz-GSSG (Figure 5). This quenching was completely reversed by the addition of PDI plus DTT, which reduced diabz-GSSG back to abz-GSH. Similar results were obtained when abz-cysteine was oxidized in the presence of Cu^{2+} to yield diabz-Cys (results not shown). These results clearly illustrate the usefulness of abz-GSH/diabz-GSSG as thiol redox probes.

One of the most important advantages of the diabz-GSSG fluorescent assay presented here is its higher sensitivity compared with two most commonly used assays. Scrambled RNase assay and insulin turbidity assay have long initial lag phases due to which the actual initial rates for enzymatic activity cannot be determined. The diabz-GSSG assay presented here shows instantaneous increase in fluorescence upon addition of PDI, thus enabling the estimation of true initial rates (Figure 6). diabz-GSSG was further used to study the single turnover of enzyme in the presence of minimal amount of DTT (Figure 9A) or in the absence of reducing agents (Figure 9B), and results obtained clearly demonstrate the sensitivity as well as its potential usefulness as a kinetic probe to explore the active-site environments of PDI and other members of the thioredoxin family of proteins.

Although fluorescence-quenched peptides have been used in the past for quantitative analysis of PDI disulphide reduction activity [12,13], with sensitivity comparable with diabz-GSSG assay presented here, the method of preparation of these disulphide-linked peptides is not straightforward [13]. The synthesis of these peptides requires expensive chemicals and a peptide synthesizer that restricts its wider acceptance as a commonly used assay for PDI activity. Moreover, the performance of the assay has not been established in crude samples. The probe presented here is synthesized in one simple step by incubating readily available chemicals, IA and GSSG. The end-product of the reaction is

always diabz-GSSG with no by-products, which makes the purification process very simple.

csPDI activity has been studied earlier using scRNase assay [25]. In the present study, we have optimized this assay to study platelet csPDI activity (Figures 10A and 10B). This method of continuously monitoring platelet csPDI activity in one step is much simpler and more sensitive than the previously described assay. The scRNase assay requires incubation of platelets with scRNase in the presence of a reducing agent that would convert a fraction of scRNase into its native active form. The sample is then centrifuged and the supernatant that contains native RNase is transferred to a solution containing its substrate RNA or CTP to monitor further the native RNase activity at 260 or 295 nm respectively. Apart from this additional step required for scRNase assay, there is always a significant blank rate associated with the process due to non-enzymatic conversion of scRNase into its native active form in the presence of DTT alone [25], which makes the process less sensitive compared with the diabz-GSSG assay. Platelet csPDI has been shown to be actively involved in disulphide isomerization [20,23] as well as denitrosation [19], which affects the process of platelet aggregation. Studies involving platelet csPDI activity are not straightforward due to lack of sensitive probes. Owing to the sensitivity of diabz-GSSG assay, the probe can be very useful for these studies. Moreover, it could be effectively used for studies involving the effect of redox buffers or reactive oxygen species on platelet csPDI activity and its consequences on platelet functions [19,20,23]. We are currently investigating these effects using diabz-GSSG assay.

In summary, in this paper, we have presented a new simple fluorescent disulphide probe for continuous detection of PDI disulphide reduction activity. Synthesis of the probe is simple, straightforward and inexpensive. The assay is rapid, sensitive and can be applied to cellular samples. The assay can be easily optimized for a fluorescence plate reader and used for high-throughput screening of PDI inhibitors from chemical libraries. The assay can be used for monitoring a single turnover of enzyme and to estimate true initial rates of disulphide reduction in crude sample preparations.

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