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Estimation of Protein Disulfide-isomerase Activity Based on Protein Refolding*

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Abstract

Protein disulfide-isomerase (PDI) is associated with the refolding of proteins that have mis-matched disulfide bonds through the thiol-disulfide interchange reaction. When the enzyme activity with reduced-denatured RNase as the substrate was plotted for analysis of the first-order kinetics, the accelerated regeneration of RNase activity gave linear plots for both the PDI concentration and reaction time. This estimation method is simple and can be used with the values which show high regeneration of RNase activity (90%), so it should be suitable for the investigation of PDI inhibitors and inhibitors of protein refolding, and for research into the refolding process of other protein substrates, such as recombinant proteins.

Key words: protein disulfide-isomerase, protein refolding, first-order kinetics, disulfide bond, RNase activity

Introduction

Protein disulfide-isomerase (PDI, EC 5.3.4.1) is a catalyst that forms native disulfide bonds of secreted proteins during protein translation[1]. This enzyme may be identical with glutathione-insulin transhydrogenase (EC 1.8.4.2)[2]. The primary structure of PDI has been identified[3] and analysis has shown that its structure is the same as that of β-subunits of prolyl 4-hydroxylase (EC 1.14.11.2)[4], thyroid hormone binding protein[5], iodothyronine-5'-monodeiodinase[6], and glycosylation site binding protein[7]. Thus, the essential physiological functions of PDI are of interest[8,9].

PDI associated with the folding of proteins, in which the inactive form of the protein is converted to the active form by formation of stable disulfide pairs. PDI can be used to refold recombinant proteins expressed and made insoluble in bacteria[10]. It could be used in both *in vivo* and *in vitro* studies of the role of PDI in the folding of various proteins, including recombinant proteins. Only a few reports on the assay of PDI activity give

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linear results, and there is no method to measure the isomerization activity accurately. The most reliable method for the assay of PDI activity would be the direct monitoring of the disulfide exchange reaction, but a suitable substrate has not been identified. Two methods are mainly used now for this assay. One is based on the detection of the splitting of insulin into its two component chains[11], and the other is based on the regeneration of enzyme activity from "scrambled" ribonuclease A (RNase), which has randomly paired disulfide bonds and is enzymatically inactive[12]. The former method does not reflect the extent of protein refolding. The latter is based on the regeneration of RNase activity, but it indirectly detects minor changes in RNase activity by the change in absorbance at 260 nm relative to that at 280 nm, and does not reflect the whole extent of RNase refolding. A method to assay PDI activity based on protein refolding is needed if PDI is to be useful for the regeneration of recombinant protein not in its native conformation. In this paper, we report an assay method that gives linear plots for PDI activity based on protein refolding by the use of the reduced-denatured and scrabmled forms of RNase.

Materials and Methods

PDI was purified from fresh bovine liver by the method of Lambert and Freedman[13]. The reaction mixture contained, in a total volume of 200 μl, 0.1 M Tris-HCl buffer (pH 7.8), 0.1 mM oxidized form of glutathione (GSSG), 200 μg/ml reduced-denatured RNase, and PDI. The reaction was started by the addition of the substrate (the RNase) and proceeded at 25°C. At times, 20-μl portions of the reaction mixture were taken and the reaction was stopped by its dilution with 0.2 M sodium acetate buffer (pH 5.0) containing 1 mg/ml bovine serum albumin (BSA, Sigma, fraction V). Each diluted sample was then assayed for RNase activity by a slightly modifed method of Anfinsen *et al*[14]. The reaction mixture for the assay contained, in a total volume of 1.0 ml, 0.1 M sodium acetate buffer (pH 5.0), 2 mg/ml RNA (sodium salt, Kohjin), 0.25 mg/ml BSA, and enzyme. The reaction was carried out at 37°C and stopped by the addition of 0.25 ml of 0.83 N perchloric acid, followed by cooling of the mixture in ice. After centrifugation at 1,700×g for 5 min, supernatant was diluted 20 times with distilled water and its absorbance at 260 nm was measured. Reduced-denatured RNase was prepared by the method described elsewhere[15] with bovine pancreatic ribonuclease A (Sigma, Type I-A) not further purified. The protein concentration was assayed at 275 nm with use of molar extenction coefficients of 9,300 and 8,100 for native and reduced-denatured RNase, respectively[16].

Results and Discussion

Figure 1 shows the course of the PDI reaction with reduced-denatured RNase as the substrate. The percentage of regeneration (y-axis) was calculated relative to the specific activity of native RNase. The regeneration of RNase activity was accelerated in proportion to the concentration of PDI. These reactions were in the presence of 0.1 mM GSSG, but the RNase was regenerated under other redox conditions, such as in the presence of both 4 mM reduced form of glutathione (GSH) and 0.4 mM GSSG (data not shown). The effects of PDI were negligible in reaction mixtures without oxidoreductive reagents.

The course of the regenerated activity was replotted in Fig. 2A for analysis of the first-order kinetics. The pattern of the regeneration of reduced-denatured trypsinogen gives a similar plot[17]. The accelerated regeneration of RNase activity gave linear plots at each PDI concentration. When the results in Fig. 2A were

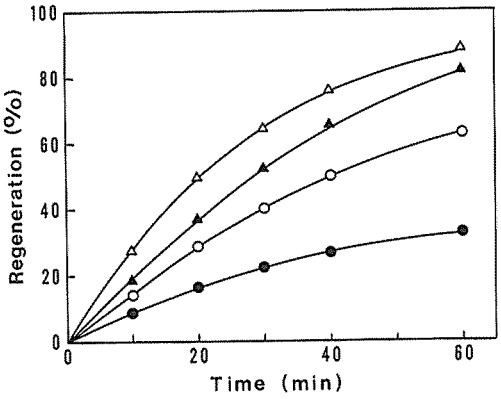

Fig. 1. Effects of PDI on the Regeneration of RNase Activity. The course of the PDI reaction with reduced-denatured RNase as the substrate is plotted. The PDI concentrations were 0 (●), 10 (○), 20 (▲), and 30 μg/ml (△). Each percentage of RNase activity was calculated relative to the specific activity of native RNase.

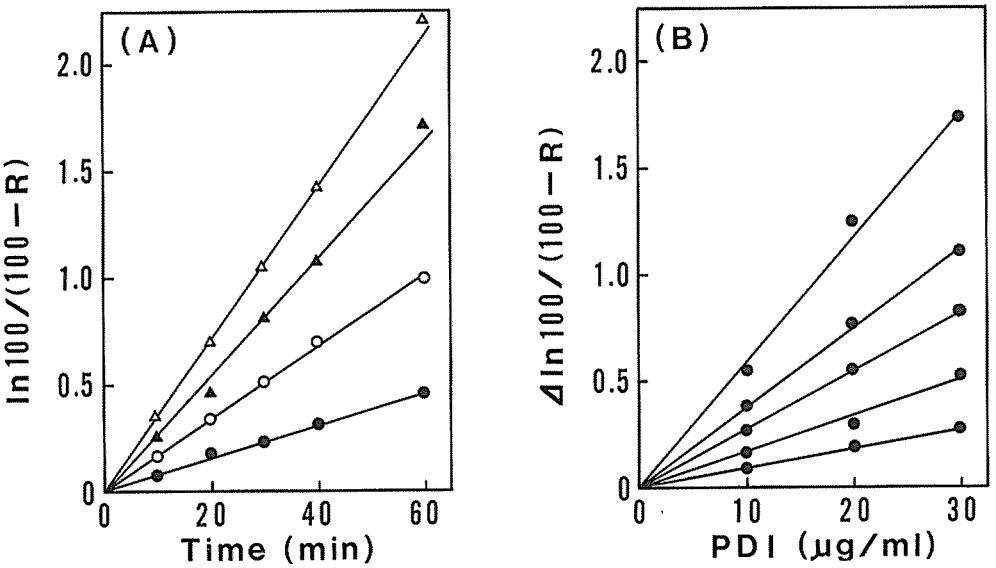

Fig. 2. First-order Kinetic Analysis of the Regeneration of RNase Activity (A) and the Dependence on the PDI Concentration (B).
(A) The data in Fig. 1 were replotted by first-order kinetics. Symbols are the same as in the legend of Fig. 1. R is the percentage of regenerated RNase activity at each point. (B) The data for the control sample (A) subtracted and the results are plotted against the PDI concentration for each reaction time. From the bottom, the lines are for reaction times of 10, 20, 30, 40, and 60 min.

plotted against the PDI concentration (Fig. 2B), the relationship was almost linear in the range of concentrations up to 30 μg/ml PDI. When the reaction time was 20 min, the range of linearity was up to at least 60 μg/ml PDI (data not shown). Moreover, the plots cover high regeneration of RNase activity (about 90%). This method for the assay of PDI activity could also be used in reaction mixtures containing scrambled RNase as a substrate, in which case dithiothreitol or GSH was added instead of GSSG and the amount of PDI used was smaller (data not shown). However, preparation of scrambled RNase takes time. The reaction should be for 20–30 min for PDI assay when reduced-denatured or scrambled RNase is used as the substrate.

The other methods[10,11] described above may be suitable for kinetic studies of this enzyme. The method

proposed here, however, is simpler, more useful, and convenient for studies on the effects of PDI on the protein refolding process. It is also suitable for comparison of the refolding ability of proteins with PDI-like activity, such as thioredoxin[15]. The method would be suitable for the investigation of PDI inhibitors and inhibitors of protein refolding, and for research into the refolding process of other protein substrates.

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タンパク質の Refolding に基づいた Protein Disulfide-isomerase 活性の評価

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Protein Disulfide-isomerase (PDI) は，thiol·disulfide 交換反応により誤った disulfide 結合を持つタンパク質の refolding を促進する。本酵素の基質として還元変性 RNase を用い，その活性の全体的な復元過程を first-order kinetics によりプロットしたところ，PDI 濃度並びに反応時間の両者について直線関係が得られた。この評価法は，簡単で，高い refolding 値（約90%）まで包括することから，遺伝子組換えタンパク質をはじめとする種々のタンパク質の refolding に対する PDI の効果の解析，並びに，PDI の阻害剤およびタンパク質の refolding に対する阻害因子の検討などに有効な方法と考えられる。