Volume 290, number 1,2, 87–89 FEBS 10170 September 1991

ADONIS 001457939100880G

Formation of enzyme–substrate disulfide linkage during catalysis by protein disulfide isomerase

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Received 4 July 1991

During the regeneration of native ribonuclease A (RNase) from the disulfide scrambled molecule by protein disulfide isomerase (PDI), the substrate forms a covalent intermediate with the enzyme through disulfide linkage(s). This has been shown by the appearance of a band at the molecular weight position expected in SDS-PAGE at the same time as the increase in RNase activity. The new band decreased when the regeneration of RNase activity approached completion and disappeared by treatment of the reaction mixture with excess dithiothreitol.

Protein disulfide isomerase; Covalent intermediate; Ribonuclease A

1. INTRODUCTION

Protein disulfide isomerase (PDI) catalyzes the inter- and intramolecular thiol-disulfide interchange reactions, thus playing an important role by facilitating the formation of native disulfide-containing proteins [1,2]. The enzyme contains both thiol and disulfide groups [3–6] and Creighton [7] suggested that PDI acts by forming mixed disulfide bonds with the substrate as intermediate. However, so far no experimental evidence has been provided. It is shown in the present study by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that during the reaction of PDI with scrambled pancreatic ribonuclease A (RNase), an additional band appeared at the expected position which disappeared in the presence of dithiothreitol (DTT) in excess.

2. MATERIALS AND METHODS

The preparation of PDI was essentially as described by Lambert and Freedman [8] with slight modifications. RNase was a Sigma product and RNase with its disulfide bonds randomly joined, scrambled RNase, was prepared by oxidation of reduced RNase in 6 M guanidine-HCl [9,10].

The reaction of PDI, 0.5 or 1 mg/ml, with scrambled RNase was carried out by first incubating PDI with 2.5 mM DTT in 50 mM Tris-HCl and 5 mM EDTA, pH 7.5, for 30 min at 30°C. To each 5 μl of the above mixture was then added 20 μl scrambled RNase, 3 mg/ml in the same buffer to start the reaction. At time intervals, the reaction mixture was mixed with 5 μl of a solution in the same buffer but 6% in SDS, 0.001% in Bromophenol blue and as indicated 12 mM in 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The mixture was rapidly shaken and heated to 100°C for a few minutes to stop the reaction. The samples were then used for SDS-PAGE with an LKB 2117 Multiphor II Electrophoresis System. The electropherogram was scanned by an UltroScan XL laser densitometer after silver staining. For activity assay of RNase, aliquots of the above reaction mixture were taken at time intervals and mixed with 3 ml of 2.5 mg yeast RNA in 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5 and the change in $A_{260\,nm}$ was followed. The unit of RNase activity was defined as by Lambert and Freedman [11].

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3. RESULTS AND DISCUSSION

The results of electrophoresis after different times of reaction of PDI with scrambled RNase are shown in Fig. 1. At time zero, the electropherogram shows bands with molecular mass (M) corresponding to scrambled RNase and the subunit of PDI, respectively. The position of scrambled and monomeric RNase was somewhat abnormal and appeared above the 14 kDa marker as discussed by Andrews [12]. At a reaction time of 3 min, a new band appeared and increased with increasing reaction time above PDI with a M of 70 kDa which is the combined M of the PDI subunit and scrambled RNase. When DTNB was not present to oxidize the excess of DTT present, the 70 kDa band was not discernible (Fig. 1B, lanes marked with *). However, when the amount of PDI, in terms of the total amount of thiol and disulfide groups, was in excess of the DTT added, the 70 kDa band was recognizable even without the addition of DTNB for the oxidation of DTT (Fig. 1A, lanes marked with *). Furthermore, the 70 kDa band was not discernible at time zero with the addition of DTNB. Thus the band corresponding to covalently linked enzyme with substrate cannot be ascribed to oxidative formation of mixed disulfide between PDI

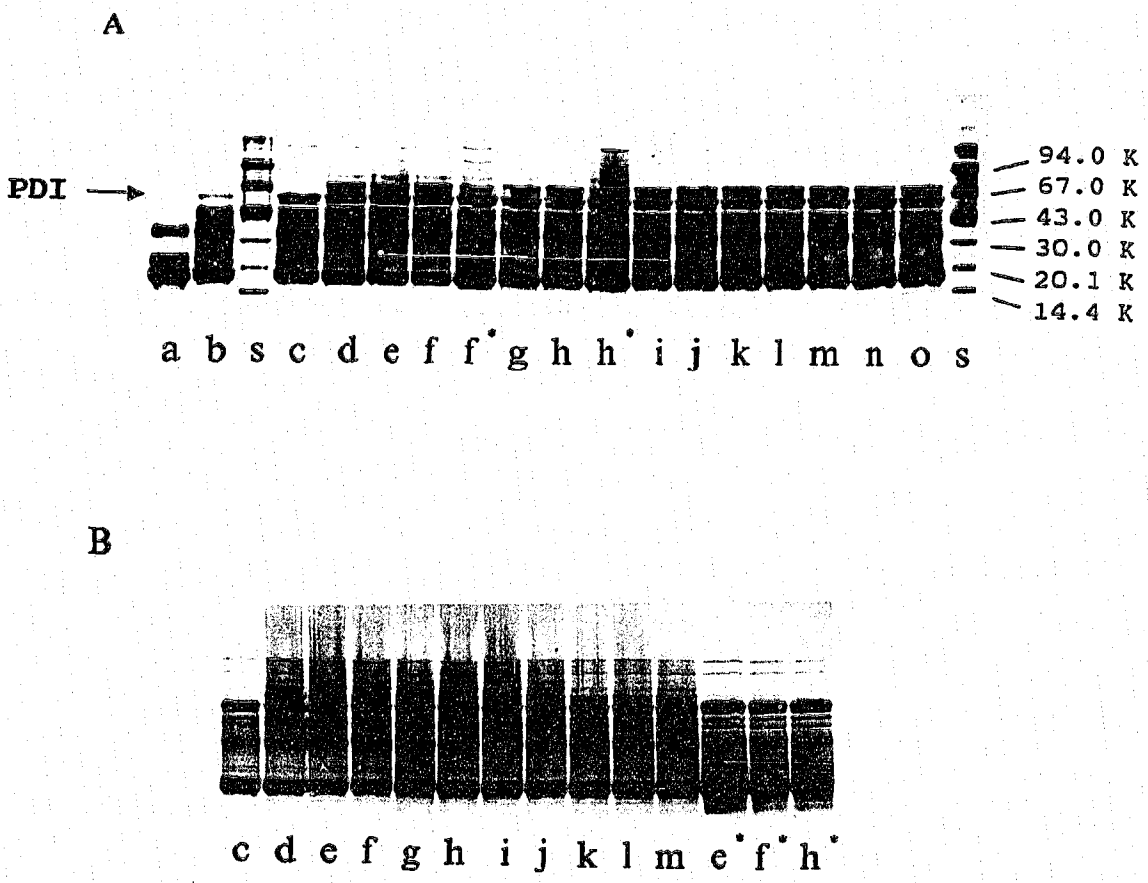

Fig. 1. SDS-polyacrylamide gel electrophoresis of the formation of mixed disulfide bridges between PDI and the substrate of scrambled RNase. (A) PDI, 1 mg/ml; (B) PDI, 0.5 mg/ml; lane a, reduced RNase; b, scrambled RNase; s, molecular weight standards; and lanes c through o represent reaction times of 0, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80 min, respectively, for both A and B. DTNB was omitted for lanes marked with *.

and scrambled RNase by DTNB after the reaction had been stopped.

At low PDI concentrations, additional bands at *M* of 82 and 91 kDa were present. The change in intensities of these bands followed similar courses as did the 70 kDa band and disappeared completely when DTT was present in excess. It is suggested that the 70 kDa species is a disulfide linked compound of the PDI subunit with scrambled RNase and it is possible that the PDI molecule may bind with more than one scrambled RNase chain. This could happen either with more than one scrambled RNase molecule linked to different thiol groups of PDI or through the same thiol group of PDI but with multiple scrambled RNase molecules themselves crosslinked through disulfides. In this connection, Freedman [1] suggested that each active site of the PDI subunit acts independently.

Fig. 2 shows the time course of the formation of the 70 kDa species in relation to the regeneration of RNase activity. It can be seen that the 70 kDa species rapidly reaches a maximum whereas the rate of formation of active RNase remains linear for the initial 10–20 min and then decreases significantly with the gradual decrease in the amount of the 70 kDa species. The above is similar to the relation of the concentration of Michaelis complex formed with the rate of an enzyme catalyzed reaction suggesting that the 70 kDa species is indeed an intermediate in PDI catalysis. In addition when the amount of PDI added was doubled, the amount of this complex formed was also approximately doubled (data not shown) as expected for its role as an intermediate in the catalyzed reaction.

Roth and Pierce [13] reported that PDI can be crosslinked to immunoglobulin by treatment in vivo with a thiol cleavable bifunctional crosslinking reagent sug-

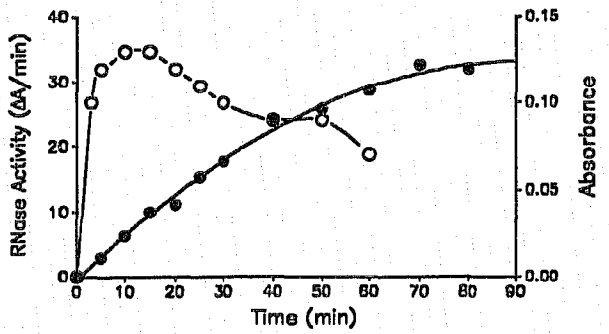

Fig. 2. The relation of PDI-scrambled RNase complex and the reactivation of scrambled RNase. (○), gel scan analysis of the PDI-scrambled RNase complex; (●), reactivation of scrambled RNase. The initial concentration of PDI was 0.5 mg/ml.

gesting that these two proteins are in close proximity and PDI may function in the in vivo synthesis of immunoglobulin. However, our results show the direct crosslinking of enzyme and substrate through mixed disulfide formation during the in vitro action of PDI.

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