ATP Binding and Hydrolysis by the Multifunctional Protein Disulfide Isomerase*

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We previously reported the ability of protein disulfide isomerase (PDI) to undergo an ATP-dependent autophosphorylation. Our efforts to map the modification site have been hindered by the low abundance and instability of the labeling. Results are presented in this paper on the nature of phospho-PDI, which appears as an intermediate with a half-life of 2.5–8.8 min in an ATPase reaction. ATP binds to PDI with high affinity, $K_d$ 9.66 μM, and the kinetic parameters $K_m$ of ATP and $K_m$ of the ATPase reaction were measured by using a pyruvate kinase-lactate dehydrogenase-coupled assay under various conditions. Strikingly, the ATPase reaction is stimulated in the presence of denatured polypeptides, while the disulfide oxidation activity of PDI is not affected by ATP. However, PDI is known to participate in various unrelated functions in the endoplasmic reticulum, and ATP could be involved in the regulation of one of these. The results are discussed in light of recent findings on ATP-chaperone relationships.

Although there is no direct experimental evidence of the presence of ATP in the lumen of the endoplasmic reticulum (ER), it has been previously established that it is required to support the correct folding and disulfide bond formation of proteins (1). Molecular chaperones of the ER, such as BiP, grp94, and calnexin, hydrolyze or at least bind ATP in the course of their activity in vitro (2), which also strongly suggests that an ATP pool should exist in the lumen in this compartment. Finally, the characterization of an ATP translocator in the ER membranes of mammalian (3) and yeast (4) cells has settled the question of how ATP accumulates in the ER. Our laboratory reported that protein disulfide isomerase (PDI), an abundant microsomal protein, can undergo an ATP-dependent autophosphorylation in vitro and likely represents another physiological target for ATP in the ER (5).

PDI was originally characterized as an enzyme (E.C. 5.3.4.1) involved in the catalysis of the protein-disulfide formation (6, 7), but in recent years, it has been found to be more than just a thioredoxin-like oxidoreductase. Its role in protein folding and cell physiology is more complex than initially thought since PDI now appears as a multifunctional protein (8) and even a chaperone (9, 10). These features could reflect the general ability of this protein to bind various peptides and proteins apparently regardless of their sequence (7, 11). PDI associates with various proteins such as the α-subunit of prolyl-hydroxylase, N-glycosyl transferase, and triglyceride transfer complex and has been revealed to be identical to the T3-binding protein, P55 (see Ref. 8 for a review).

Interestingly, it has been found by using a genetic approach in yeast that the essential function of PDI does not reside in the thioredoxin-like domains (12). In this context, we previously suggested that PDI phosphorylation modulates its interaction with various partners (5). The precise relationship between ATP and PDI remains elusive. Preliminary attempts to find the effect of ATP on PDI catalysis (13) or ATP hydrolysis (14) were unsuccessful. Using affinity chromatography on various immobilized denatured proteins, Nigam et al. (15) reported the Ca$^{2+}$-dependent association of several ER proteins, among them PDI, with these substrates and their elution by ATP. However, the experiments did not provide a direct association between PDI and ATP.

The following paper presents the data we obtained on ATP binding by PDI and the use of a sensitive spectrophotometric method to demonstrate the existence of an ATPase activity for this protein. The kinetic parameters of this activity were measured under various conditions. The results suggest a new role for ATP in the ER, which uses the multifunctional PDI as a target.

EXPERIMENTAL PROCEDURES

Enzyme Preparations—Recombinant human PDI (rh-PDI) was obtained from an Escherichia coli strain BL21(DE3) transformed with the plasmid pTM2-PDI (kindly provided by Prof. K. Kivirikko, University of Oulu, Finland), as described previously (16). DsbA was obtained from an E. coli K10 strain transformed with the plasmid pPB2190, which was built by introducing the BspHI-SiPl dsb-containing cassette of pBP2212 (17) into the expression vector pTrc99A (Pharmacia Biotech Inc.). E. coli thioredoxin was purchased from Promega. All proteins used in this study were controlled and eventually repurified by HPLC using a TSK 3000SW (TosoHaas) gel filtration column (flow rate, 1 ml/min; mobile phase, 20 mM sodium phosphate; 0.3 mM NaCl, pH 7.4). They were more than 95% pure when analyzed by SDS-PAGE.

Measurement of the Phospho-PDI Half-life—$100 \mu g$ of rh-PDI was pulse labeled for 5 min at 37°C in 0.5 ml of 0.5 mM Tris acetate, 10 mM magnesium chloride, 0.15 mM NADH, 6 μM NADH, 0.2 mM GSSG and 60 μM GSH, containing 1 mM GSH, 0.2 mM GSSG with 60 μCi of [γ-32P]ATP (final concentration, 40 nM). The mixture was separated into two samples, which were complemented with either 1 mM unlabeled ATP or nothing. At various times, 20-μl aliquots were removed, and the reaction was stopped by instant freezing in liquid nitrogen. The phospho-PDI in each sample was separated by SDS, 10% PAGE (1 μg per well) and quantitated by densitometry of the autoradiogram.

Coupled ATPase Assay—Continuous spectrophotometric measurement of the ATP conversion into ADP was performed in a pyruvate kinase-lactate dehydrogenase-coupled assay. $100 \mu g$ of rh-PDI (final monomer concentration, 1.8 μM) was allowed to equilibrate at 30°C for 5 min in 1 ml of 0.1 mM triethanolamine, pH 7.5, 0.16 mM potassium chloride, 50 mM magnesium sulfate, 0.25 mM phosphoenolpyruvate, 0.15 mM NADH, 6 μg/ml pyruvate kinase, 2 μg/ml lactate dehydrogenase. When indicated, 1 mM GSH, 0.2 mM GSSG, or 100 μg of denatured
RNase-A, either reduced or alkylated with N-ethylmaleimide, was added. The reaction was started by addition of ATP (1–100 μM) and monitored at 340 nm for 15 min.

PDI-mediated RNase A Refolding—The assay was carried out essentially as already described (18) with minor modifications. Ribonuclease A (Sigma) was denatured at 10 mg/ml in 0.1 M Tris acetate, pH 8.0, 0.1 mM DTT, 6 M guanidine chloride and then dialyzed overnight against 0.1 M PDI in 0.1 M Tris-HCl, pH 8. The residual 30 mM guanidine chloride has no effect on the PDI activity. The concentration of active RNase A was at each time was calculated using the equation and constants reported in Ref. 18 but modified to take into account the inhibitory effect of ATP on RNase activity (Equation 1). The inhibition constant for ATP \( K_{iATP} \) was determined at 0.8 μM.

\[
C_a = \frac{[\text{ATP}]_o}{K_{iATP} \left[ \frac{1}{K_{\text{ATP}}} + \frac{[\text{CMP}]}{K_{\text{CMP}}} + \frac{[\text{cCMP}]}{K_{\text{cCMP}}} \right]} \quad \text{Eq.} \ 1
\]

ATP Binding by Equilibrium Dialysis—Experiments were performed with an equilibrium microdialysis (EMD 101, Hoefer Scientific Instruments). 24 chambers, mounted around 12–14-kDa cut-off membranes. The wells were filled with 60 μg of rh-PDI on one side of the membrane, [\( ^{32} \text{P} \)]ATP on the other side, and then filled up to 60 μl with 0.1 M Tris-HCl buffer, pH 7.5. The system was rotated overnight at room temperature, and the concentrations of bound and free ligand were estimated by liquid scintillation counting on 50-μl samples from both sides of the chambers.

RESULTS

The ability of PDI to autophosphorylate in the presence of \( [\gamma-^{32} \text{P}] \text{ATP} \) has been shown previously (5). However, the phospho-PDI obtained in such conditions turned out to be very unstable, rendering it very difficult to handle for further experiments such as mapping of the phosphorylation site. The transient nature of the phosphorylation was quantitatively approached, and half-lives of 2.5 and 8.8 min were measured under ATP chase or without chase conditions, respectively (Fig. 1). The maximal quantity of \( ^{32} \text{P} \) radioactivity incorporated into PDI indicated that phospho-PDI never accumulates to more than 0.4%. The substrate specificity of the phosphate donor was investigated, and we observed that both \( [\gamma-^{32} \text{P}] \text{ATP} \) and \( [\gamma-^{35} \text{S}] \text{ATP} \) can be substituted for \( [\gamma-^{32} \text{P}] \text{ATP} \) without any apparent modification in the labeling efficiency. On the other hand, [\( \alpha-^{32} \text{P} \)]ATP, [\( \beta-^{35} \text{S} \)]JADP, [\( 5^- \text{S} \)]JAMP, or [\( 3^2 \text{P} \)]IP\( ^3 \) did not give rise to PDI labeling (data not shown). These preliminary data suggested that phospho-PDI forms by a reaction involving the \( \gamma \)-phosphoryl of ATP or dATP and requires cleavage of the bond between the \( \gamma \) - and \( \beta \)- phosphates. The presence of Mg\( ^{2+} \) in the phosphorylation mixture is critical for the formation of phospho-PDI; therefore, the substrate of the reaction is certainly the Mg-ATP complex.

The properties of this ATPase activity of PDI were assayed in a pyruvate kinase-lactate dehydrogenase-coupled reaction. A significant deviation of the absorbance at 340 nm was observed in presence of PDI, which implies a significant consumption of ATP (Fig. 2). Prokaryotic oxidoreductases related to PDI, i.e. thioredoxin and DsbA, are devoid of this activity. This is in good agreement with our preliminary report that the site of phosphorylation, and thus probably the ATPase active site, lies somewhere within the central domain of the protein (5), which is specific for the mammalian enzyme. This site is far away from the redox active sites in the sequence. Furthermore, the measurements of the rates of PDI-catalyzed refolding of denatured-reduced RNase A in the absence \((0.70 \pm 0.02 \text{ mmol RNaseA} \cdot \text{min}^{-1} \cdot \text{mmol PDI}^{-1})\) or in the presence of ATP \((0.70 \pm 0.02 \text{ mmol RNaseA} \cdot \text{min}^{-1} \cdot \text{mmol PDI}^{-1})\) show that ATP does not, or only very slightly, affects this PDI activity (Fig. 3). We are thus in agreement with a previous report describing the lack of direct effect of ATP on the disulfide formation catalysis (13).

However, when we measured the kinetic parameters of this ATPase reaction under various conditions, we saw an unexpected dependence of this parameter on the assay conditions. While the rate of hydrolysis is 0.057 μmol min\(^{-1} \cdot \mu\text{mole}^{-1}\) for PDI alone, it increases 6.7-fold when the hydrolysis is measured under the conditions of the RNase refolding assay, i.e. in the presence of GSH, GSSG, and RNase A (Fig. 4). A comparison of various effectors was carried out to clarify how the PDI-ATPase depends on assay conditions (Table I). The redox state of the medium does not seem to be the basis of this effect since GSH, GSSG, or DTT does not allow the hydrolysis to reach the optimal value. Nevertheless, both GSH and GSSG lead to a significant 2-fold increase. The presence of denatured RNase A, either alone or associated with the redox partners GSH and GSSG, enhances the ATPase 5–7-fold without significant alteration of the \( K_m \). The alkylation of the eight RNase thiol groups with N-ethylmaleimide did not alter its stimulatory effect on the hydrolysis. Furthermore, a control experiment performed with lysozyme displayed similar activity. The
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**TABLE I**

Kinetic parameters of the PDI ATPase dependence on the assay conditions

| Effector in the ATPase assay | \( V_m \) | \( K_m \) |
|------------------------------|----------|----------|
| rh-PDI                       |          |          |
| None                         | 0.057 ± 0.007* | 11.64 ± 4.33* |
| GSH, GSSG, dr-RNase A        | 0.384 ± 0.024* | 4.33 ± 0.83* |
| GSH, GSSG, da-RNase A        | 0.371*     | ND       |
| GSH                          | 0.126 ± 0.018* | 5.8 ± 1.04* |
| DTT                          | 0.068*     | ND       |
| GSSG                         | 0.154*     | ND       |
| da-RNase A                   | 0.326*     | ND       |
| da-lysozyme                  | 0.283*     | ND       |
| bPDI                         | 0.063*     |          |
| GSH, GSSG, dr-RNase A        | 0.378 ± 0.0034* | 5.05 ± 1.47* |

*Results given when kinetic data are available over a large ATP range.

**DISCUSSION**

These results describe a new property for the multifunctional protein PDI. Both ATP binding and hydrolysis properties are clearly exhibited and were extensively characterized. The data presented here corroborate the preliminary evidence published in our first paper on bovine liver PDI (5). Several soluble and membrane-bound ATPases have been found in microsomal extracts that could contaminate our enzyme preparations. We ruled out this possibility for the following reasons: similar \( V_m \) and \( K_m \) were measured for both human recombinant PDI expressed in E. coli and for the bovine PDI extracted from ox liver microsomes (Fig. 4, Table I), the calculation of the concentra-

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**Fig. 3. Effect of ATP on the PDI-catalyzed RNase A refolding.** A, the rh-PDI-catalyzed formation of native RNase A is followed from its enzymatic activity recovery, i.e. hydrolysis of 2',3'-cyclic CMP into 3'-CMP in the absence (●) or in presence (○) of 0.1 mM Mg-ATP. The amount of active RNase at each time (t) was calculated from \( A_{396\text{nm}} \) versus time as described under "Experimental Procedures." PDI activity is the slope of the regression in the linear phase of the reaction and after a 2-3 min lag. Non-catalyzed RNase renaturation was subtracted in the activity calculations.

**Fig. 4. Kinetic analysis of the PDI ATPase activity.** ATP hydrolysis was estimated from the pyruvate kinase-lactate dehydrogenase-coupled assay at various ATP concentrations. Kinetic parameters \( K_m \) and \( V_m \) were determined from the fitting of the data to a Michaelis-Menten curve (A) for rh-PDI alone (●) and for rh-PDI (○) and bovine PDI (□) in the presence of GSH, GSSG, and RNase in the assay mixture. The cooperativity under optimal conditions of ATP hydrolysis by rh-PDI (○) was assessed from a Hill plot (B).
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The presence of peptide substrate in the assay increases the rate of hydrolysis while ATP by itself does not promote the apparent activity of PDI on RNase refolding. In some respects, the situation could be close to that of Hsc70-ATPase, which is stimulated 2–3-fold upon addition of an unfolded polypeptide (21). There is no apparent stoichiometry between ATP hydrolysis and S–S bridge formation. From a thermodynamic point of view, disulfide bond oxidization is exergonic and thus, a priori, does not require the energy input provided by the ATP hydrolysis. In the case of DnaK, it was clearly demonstrated that the ATPase is not stoichiometrically coupled to a peptide binding/release cycle (22, 23). The requirement for ATP hydrolysis is not even absolute in the chaperone function of either GroEL (24) or DnaK (25). Both ATP and ADP are able to change the conformation of GroEL (26, 27), whose protein binding properties are also regulated by heat shock-induced phosphorylation (28). The relationship between PDI and ATP is puzzling and might reveal another possible likeness with molecular chaperones. However, we regard this with extreme reservation and we are at work on it to validate this presumption. The function of PDI, which is possibly under ATP control, and the role of hydrolysis remain to be determined as well as whether the conclusions drawn in vitro are also relevant in the cellular context.

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