Two resident ER-proteins, CaBP1 and CaBP2, with thioredoxin domains, are substrates for thioredoxin reductase: comparison with protein disulfide isomerase

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Abstract Protein disulfide-isomerase (PDI) is the best known representative of a growing family of enzymes with thioredoxin domains. Two such proteins with thioredoxin (Trx) domains, CaBP1 and CaBP2 (ERp72), have previously been isolated from rat liver microsomes. Here we report that they, like PDI are substrates for thioredoxin reductase and will catalyze NADPH-dependent insulin disulfide reduction. The activity of CaBP1 and CaBP2 in this assay was higher than that of PDI but lower than that of *E. coli* Trx. Furthermore, as isolated the thioredoxin domains of CaBP1 and CaBP2 were in disulfide form as judged by stoichiometric oxidation of 2 and 3 mol of NADPH in CaBP1 and CaBP2, respectively. The redox potential of the active site disulfide/dithiol was estimated from the equilibrium with a mutant *E.coli* Trx, P34H Trx, with a known redox potential (−235 mV). This showed that CaBP1 and CaBP2, like PDI, have a much higher redox potential than wild type thioredoxin (−270 mV) in agreement with a role in formation of protein disulfide bonds. In conclusion, in vitro CaBP1 and CaBP2 share catalytic properties in thiol disulfide-interchange reactions with PDI. Thus, the well known activity of PDI is not unique in the endoplasmic reticulum and CaBP1 and CaBP2 may be regarded as functional equivalents.

Key words: Protein disulfide-isomerase; Thioredoxin; Thioredoxin reductase; Protein folding; Endoplasmic reticulum; Redox potential

1. Introduction

Protein disulfide-isomerase (PDI, M_r 57,000) was originally isolated as a sulfhydryl-interchange enzyme from beef liver microsomes based on its ability to catalyze formation of native disulfides and folding of reduced ribonuclease [1]. It can also degrade insulin in a GSH-dependent manner and has a historical identity as GSH-disulfide transhydrogenase. Studies on the role of PDI in disulfide formation have been summarized by Freedman [2]. Cloning and sequencing cDNA for PDI revealed that PDI contains two domains with strong sequence homology to thioredoxin (Trx) [3]. Thioredoxin has a redoxactive disulfide in the sequence CGPC, which, in its known three-dimensional structure is located in a hydrophobic surface area and catalyzes dithiol-disulfide oxidoreduction of protein disulfides [4–6]. The Trx domains of PDI differ in active site sequence by a Pro to His substitution, but have apparent catalytical properties in common with Trx, including being substrates for thioredoxin reductase [7–9]. However they show a higher redox potential (−190 or −175 mV), which at least in part is due to the Pro to His replacement [10,11].

Recently, additional proteins from the endoplasmic reticulum have unexpectedly been shown to contain similar Trx-like sequences. CaBP2 (ERp72) contains three domains with homology to Trx and was isolated on the basis of its calcium binding [12] and stress response properties [13]. CaBP1 contains two Trx-like domains in closer proximity when compared to PDI and was isolated for its calcium binding propererties [14] or upregulation in hydroxyurea resistant cells [15]. We have previously shown that CaBP1 and CaBP2 display protein disulfide reductase activity with insulin in the presence of a low molecular weight thiol. Furthermore, they catalyzed formation of protein disulfide bonds in reduced ribonuclease or an Fab fragment [16].

Trx or PDI catalyzes reduction of insulin disulfides together with thioredoxin reductase and NADPH in the following reactions [4,9].

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \overset{\text{TR}}{\rightleftharpoons} \text{Trx-(SH)}_2 + \text{NADP}^+ \quad (1)$$

$$\text{Trx-(SH)}_2 + \text{insulin-S}_2 \rightleftharpoons \text{Trx-S}_2 + \text{insulin-(SH)}_2 \quad (2)$$

In this study we examine the ability of CaBP1 and CaBP2 to serve as substrates for thioredoxin reductase and estimate their redox potential in comparison to thioredoxins.

2. Materials and methods

DTT, NADPH , $NADP^+$, DTNB and sodium iodoacetate were purchased from Sigma. Insulin was a gift from from Nordisk Novo. [^{14}C]Iodoacetate was from Amersham. All other chemicals were of analytical grade or better.

2.1. Enzyme purifications

The enzymes used in this investigation were prepared to homogeneity according to previously published procedures: CaBP1 and CaBP2 [17], *E. coli* Trx and P34H Trx [18], PDI [9] and thioredoxin reductase [19]. Concentrations of CaBP1 and CaBP2 and of PDI were determined with Commassie dye binding [20]. The concentrations of *E.coli* Trx and PDI were determined by absorbance at 280 nm, using extinction coefficients

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Abbreviations: PDI, protein disulfide-isomerase; CaBP1, calcium binding protein 1 from rat liver microsomes; CaBP2, calcium binding protein 2 from rat liver microsomes; DTT, dithiothreitol; DTNB; 5,5′-dithiobis(2-nitrobenzoic acid); Trx, *E. coli* thioredoxin; P34H Trx, *E. coli* thioredoxin with Pro-34 mutated to His; TR, thioredoxin reductase; E_0', redox potential

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of 47,300 $M^{-1} \cdot cm^{-1}$ for PDI and 14,300 $M^{-1} \cdot cm^{-1}$ for Trx. The concentration of calf thymus thioredoxin reductase (M_r 116,000) was determined by activity in a DTNB reduction assay in which 1200 A_{412}/min in 1 ml was assumed to correspond to 1 mg of the enzyme [19].

2.2. Thioredoxin reductase and NADPH-dependent insulin reduction assay

The sensitive insulin disulfide reduction assay [19] was used where formation of thiols in insulin was measured spectrophotometrically with DTNB in the presence of guanidine hydrochloride. The incubation mixture contained 30 nM TR, a defined concentration of thioredoxin, PDI, CaBP1 or CaBP2, 0.3 mM insulin, 0.7 mM NADPH, 86 mM HEPES buffer, pH 7.9 and 3.3 mM EDTA in a final volume of 0.12 ml. After incubation for 20 min at 37°C, the reaction was stopped by addition of 0.50 ml of 6 M guanidinium hydrochloride and SH-groups were determined spectrophotometrically at 412 nm.

2.3. Reduction of disulfides in CaBP1 and CaBP2 by thioredoxin reductase and NADPH

In four separate experiments a microcuvette contained either 20 μM Trx, 7.5 μM PDI, 7.5 μM CaBP1 or 7.5 μM CaBP2 and 25 μM NADPH in 0.10 M Tris-HCl, pH 7.4, 2 mM EDTA in a final volume of 0.150 ml. The absorbance at 340 nm was determined against a blank containing only buffer. Oxidation of NADPH was initiated by addition of 1 μl of a 20 μM stock solution of bovine TR (final concentration 133 nM). The decrease in absorbance was determined and the concentration of disulfides that were reducible by NADPH was calculated using a molar extinction coefficient of 6,200 $M^{-1} \cdot cm^{-1}$ for NADPH. The reversibility of the reaction was tried by addition of $NADP^+$ to a final concentration of 1 mM.

2.4. Reduction disulfides in CaBP1 and CaBP2 by reduced P34H Trx

P34H Trx was fully reduced by incubation with 5 mM DTT followed by desalting on a Sephadex G-25 column (NAP5 Pharmacia Biotechnology) in 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA. The concentration of SH-groups was determined with DTNB [21] and related to the protein concentration. The reduced Trx was then incubated at a molar ratio of 2:1 with either CaBP1, CaBP2 or PDI (30 μg each) for 20 min at 37°C in a volume of 100 μl of 100 mM potassium phosphate buffer pH 7.0 and 1 mM EDTA. Free SH-groups were alkylated during 15 min at 25°C with 1.25 mM [^{14}C]iodoacetate and 3.4 M guanidinium hydrochloride at pH 8.0. P34H Trx was purified by size fractionation on an FPLC column of Superdex-75 (Pharmacia LKB Biotechnology) and the redoxstate was determined by relating radioactivity content to protein concentration as described previously [11].

2.5. Antibodies and Western blots

Antibodies against rat liver thioredoxin reductase were raised in a rabbit by several injections of 50 μg of homogeneous protein with Freund's adjuvant (J.L. and A.H., to be published). The IgG-fraction was further purified by precipitation in 37% ammonium sulphate followed by chromatography on thioredoxin reductase immobilized on CNBr-activated Sepharose.

Cytosolic and microsomal fractions of rat liver prepared according to [17], were separated on 10% SDS-polyacrylamide by electrophoresis followed by electrical transfer to a nitrocellulose membrane. The presence of thioredoxin reductase was visualized according to standard procedures. Briefly, the nitrocellulose membrane was incubated with affinity-purified IgG against thioredoxin reductase followed by goat-antirabbit IgG conjugated with peroxidase and subsequently with 0.24 mg/ml of *N'N'*-diethyl-*p*-phenylenediamine in 100 mM sodiumcitrate, 0.3% chloronaphtol, 0.5% H_2O_2 and 1.6% CH_3CN.

3. Results

3.1. CaBP1 and CaBP2 are substrates for thioredoxin reductase

It was previously shown that CaBP1 and CaBP2 like PDI display insulin reductase activity in the presence of low molecular weight thiols like GSH, DTT or cysteamine [12,14]. The thioredoxin-like domains of PDI are substrates for thioredoxin reductase and can also reduce insulin disulfides in an NADPH-dependent manner together with bovine thioredoxin reductase

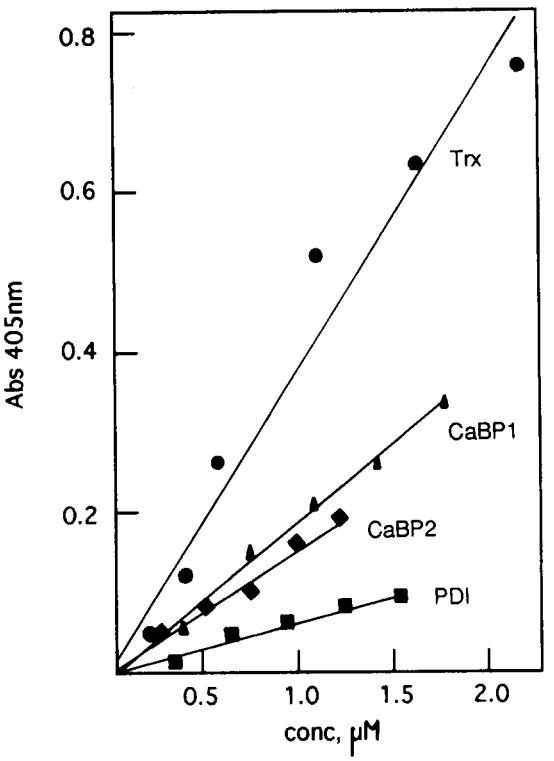

Fig. 1. Activity of CaBP1 and CaBP2 in the standard insulin assay compared to thioredoxin and PDI. PDI (■), CaBP1 (▲), CaBP2 (♦), or *E. coli* Trx (●) were assayed for their ability to catalyze NADPH-dependent insulin disulfide reduction. After 20 min incubation at 37°C, in the presence of 30 nM bovine TR, 700 μM NADPH, 300 mM insulin and 86 mM HEPES, pH 7.6 and 1 mM EDTA, the formation of SH-groups was measured with DTNB according to Ellman, 1959 (in this experiment at 405 nm).

(the insulin assay) [9]. We measured the activity of CaBP1 and CaBP2 for their ability to serve as substrates for thioredoxin reductase. As shown in Fig. 1, on a molar basis, CaBP1 was 3.0 times, CaBP2 2.6 times and *E. coli* thioredoxin was in turn 6.5-fold more active than PDI. For proteins with a high second order rate constant for the reduction of insulin [22], this assay will be an indirect measure of the interaction with thioredoxin reductase because of the high concentration of insulin (0.3 mM). A similar reaction as for PDI was previously seen for CaBP1 and CaBP2 in the reduction of 0.16 mM insulin with different low-molecular weight thiols [12,14]. Thus, the relative rates in Fig. 1 are likely to reflect different K_m-values for thioredoxin reductase.

3.2. Stoichiometry of NADPH oxidation in reduction of disulfides in CaBP1 and CaBP2

The numbers of disulfides in CaBP1 and CaBP2 that are substrates for thioredoxin reductase were measured by following oxidation of NADPH at 340 nm. Using 7.5 μM PDI, 15 μM of NADPH was oxidized (Table 1). The result was similar for CaBP1. However, in the presence of 7.5 μM CaBP2, 23 μM NADPH was oxidized corresponding to 3 mol of disulfides per mol of protein. When the reaction had reached a stable level, 1 mM $NADP^+$ was added to the cuvette to test for the reversibility of the reaction. This did not result in formation of NADPH. Controls with *E. coli* Trx resulted in the expected

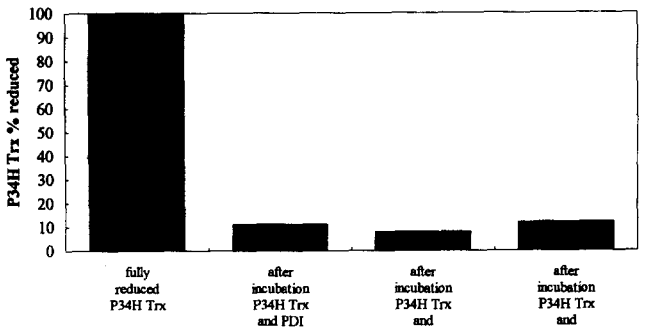

Fig. 2. Redox state of P34H Trx after incubation with PDI, CaBP1 or CaBP2. The redox state of P34H Trx was determined after incubation at a molar ratio of 2:1 of P34H Trx and PDI, CaBP1 or CaBP2, respectively. Free SH-groups were determined using [^{14}C]iodoacetate. The percent of reduced P34H Trx was calculated from the incorporation of radioactivity.

reversible reduction of one disulfide per molecule. This indicates that CaBP1 and CaBP2, like PDI, have a higher redox potential than thioredoxin [11].

The amino acid sequences of CaBP1, CaBP2 and of PDI reveal that all three proteins contain six cysteine residues [3,12,14]. In PDI and CaBP1 four of the cysteines are present in two sequence motifs homologous to the active site of thioredoxin, whereas in CaBP2 all six cysteines are present in thioredoxin-like sequences. The number of cysteine residues in the sequence CGHC are thus identical to the number of disulfides that were reducible with NADPH and thioredoxin reductase. This proves that the cysteine residues in thioredoxin-like sequences are isolated as disulfides and that these disulfides can be converted to dithiols by NADPH and thioredoxin reductase.

3.3. *Reduction of disulfides in CaBP1 and CaBP2 by P34H Trx*

The active site disulfides in PDI can be reduced by Trx and P34H Trx in a reversible mannner. The equilibrium of this reaction was previously used to determine the redox potential of the active site disulfides in PDI [11]. A limited set of experiments were performed to determine whether the redox potential of the disulfides in CaBP1 and CaBP2 are in the same range as PDI. Reduced P34H Trx was incubated together with PDI, CaBP1 or CaBP2 at a molar ratio of 2:1. Analysis of the redox state of the P34H Trx at equilibrium revealed that it had become almost completely oxidized (Fig. 2) and incorporation of iodoacetic acid into PDI (CaBP1, CaBP2) increased accordingly (data not shown). The E_0' of P34H Trx is −235 mV compared to −270 mV of wt Trx and −190 mV for PDI. We conclude that the equilibrium of the reactions:

$$\text{P34H-(SH)}_2 + \text{CaBP1-S}_2 \rightleftarrows \text{P34H-S}_2 + \text{CaBP1-(SH)}_2$$

$$\text{P34H-(SH)}_2 + \text{CaBP2-S}_2 \rightleftarrows \text{P34H-S}_2 + \text{CaBP2-(SH)}_2$$

are shifted to the right and that the two calcium binding proteins have a higher redox potential than P34H Trx.

3.4. *Cellular localization of thioredoxin reductase*

Immunoblotting with antithioredoxin reductase antibody of cytosolic and microsomal fractions of rat liver revealed exclusive staining of the cytosolic fractions. Control immunostaining with anti-CaBP1 and CaBP2 antibodies gave the expected staining of the endoplasmic reticulum fractions (data not shown here).

4. Discussion

During preparations of PDI, we have previously noted the presence of other proteins than PDI or thioredoxin that acted as substrates for thioredoxin reductase [9]. Here, we report that CaBP1 and CaBP2 are related to PDI not only by sequence but also on the basis of their mechanistic properties. Like PDI, the two proteins can catalyze protein disulfide formation, reduction and isomerization, depending on the imposed redox potential [12,16]. Furthermore, the Trx-like domains must have a similar structure compared to thioredoxin since they can serve as substrates for thioredoxin reductase and catalyze NADPH dependent insulin disulfide reduction.

PDI, CaBP1 and CaBP2 showed similar catalytic properties. On a molar basis, CaBP1 was most efficient in catalyzing reduction of insulin disulfides with NADPH and thioredoxin reductase, followed by CaBP2 and PDI. The order of efficiency is exactly the opposite (PDI>CaBP2>CaBP1>Trx) in two independent disulfide formation assays using reduced, denatured ribonuclease or Fab-fragment [16]. All three assays are complicated and depend on multiple steps. It is impossible to assess differences in activity to any particular feature of PDI or CaBP1 or CaBP2. When precipitation of insulin was followed using low molecular weight thiols the order of efficiency was the same as in the protein disulfide formation assays. Precipitation of insulin is thought to be caused by free B-chain aggregates that become insoluble at high concentration. Therefore, although reduction of disulfides in insulin is required for precipitation, this is at the same time a measure of isomerase activity.

The intracellular concentration of PDI in the ER is suggested to be very high or several hundred μM or nearly mM [23] and the concentration of CaBP1 and CaBP2 is similar (data not shown). In addition there is yet another protein with thioredoxin-like domains in the endoplasmic reticulum, ERp61, previously known as phopholipaseC-α [24]. Together, the thioredoxin-like domains in these proteins constitute a significant and catalytically active dithiol redox buffer of similar concentration as the monothiol GSH and its oxidized form GSSG. The fact that these domains can serve as substrates for thioredoxin reductase, leads to the attractive hypothesis that this protein can transfer electrons to $NADP^+$ during the oxidative formation of protein disulfides. However, this can be excluded on the basis of the intracellular location of thioredoxin reductase. If an enzyme with a similar activity is located in the endoplasmatic reticulum, an efficient NADPH consuming system would have

Table 1
Number of disulfides reducible with NADPH and thioredoxin reductase in Trx, PDI, CaBPI and CaBP2

Protein	mol SS/mol protein
Trx	1.0
PDI	2.0
CaBP1	2.0
CaBP2	3.1

The number of disulfides (S–S) was calculated from the A_{340} nm (see experimental procedures).

to be postulated to allow for the formation of disulfides. This reaction would otherwise be blocked by the more than 100 mV lower redox potential of the NADPH/$NADP^+$ couple compared to PDI (CaBP1, CaBP2).

Overexpression of either mammalian ERp72 or the gene product EUG1, rescues yeast from the otherwise lethal mutation in the PDI gene [25,26]. Although this proves that ERp72, here called CaBP2, and PDI have overlapping functions also in vivo, the picture is complicated by the fact that the essential function of PDI does not seem to reside in its disulfide isomerase activity. La Mantia have constructed different strains of yeast with mutations in the PDI gene [26]. In particular, when one cysteine in each active site was replaced by serine, disulfide formation activity was only slightly impaired. It is likely that CaBP1 and CaBP2 constitute a sufficient back-up system for the disulfide formation activity of PDI. In addition, it seems likely that PDI, as well as CaBP1 and CaBP2 could play chaperone functions [27]. In this respect, the three proteins might interact with different nascent secretory proteins.

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