The Role of Calcium on the Activity of ERcalcistorin/Protein-disulfide Isomerase and the Significance of the C-terminal and Its Calcium Binding

A COMPARISON WITH MAMMALIAN PROTEIN-DISULFIDE ISOMERASE*

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ERcalcistorin/protein-disulfide isomerase (ECaSt/PDI) shows a 55% identity with mammalian protein-disulfide isomerase (PDI) (Lucero, H. A., Lebeche, D., and Kaminer, B. (1994) J. Biol. Chem. 269, 23112–23119) is a high capacity low affinity Ca2+-binding protein and behaves as a Ca2+ storage protein in the ER of a living cell (Lucero, H. A., Lebeche, D., and Kaminer, B. (1998) J. Biol. Chem. 273, 9857–9863). Here we show that recombinant ECaSt/PDI bound 26 mol of Ca2+/mol and a C-terminal truncated mutant bound 14 mol of Ca2+/mol, both with a Kd of 2.8 mM in 50 mM KCl and 5.2 mM in 150 mM KCl. The percentage reduction in Ca2+ binding in the mutant corresponded with the percentage reduction of deleted pairs of acidic residues, postulated low affinity Ca2+-binding sites. 5 mM Ca2+ moderately increased the PDI activity of both ECaSt/PDI and the C-terminal truncated mutant on reduced RNase and insulin. Surprisingly, ECaSt/PDI in the absence of Ca2+ prevented the spontaneous reactivation of reduced bovine pancreatic trypsin inhibitor. In the presence of 1–5 mM Ca2+ (or 10 mM polylsine) ECaSt/PDI augmented the bovine pancreatic trypsin inhibitor reactivation rate. In contrast, the C-terminal truncated ECaSt/PDI augmented rBPTI reactivation in the absence of Ca2+ and 1–5 mM Ca2+ further accelerated the reactivation rate, responses similar to those obtained with mammalian PDI.

The endoplasmic reticulum (ER) is a multifunctional organelle involved in post-translational modification of nascent proteins, in lipid synthesis and regulation of intracellular Ca2+. Protein folding in the ER associated with disulfide bond formation is likely to be catalyzed by protein-disulfide isomerase (PDI), the identification of which stemmed from the early findings of a factor which catalyzed the renaturation of ribonuclease in a non-stoichiometric manner (10) and estrogen finding of a factor which catalyzed the renaturation of ribonuclease (PDI), the identification of which stemmed from the early formation is likely to be catalyzed by protein-disulfide isomerase (EC 5.3.4.1); rBPTI, reduced bovine pancreatic trypsin inhibitor; nBPTI, native bovine pancreatic trypsin inhibitor; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; CaBP, calcium-binding protein.

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† The abbreviations used are: ER, endoplasmic reticulum; PDI, protein-disulfide isomerase (EC 5.3.4.1); rBPTI, reduced bovine pancreatic trypsin inhibitor; nBPTI, native bovine pancreatic trypsin inhibitor; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; CaBP, calcium-binding protein.

A sixth domain in the C-terminal was recently labeled c as a putative Ca2+-binding domain (4). This speculation is in keeping with our findings and hypothesis on calcium binding. We found quantitatively, for the first time, that sea urchin (20) and mammalian PDI (21) are high capacity low affinity Ca2+-binding proteins. And it should be recognized that calcium binding by mammalian PDI was not quantitated previously (22). Having determined the number of calcium ions bound, we related them to the number of paired acidic residues and hypothesized that paired acidic residues constituted low affinity Ca2+-binding sites, a cluster of which resides in the tail end of the C-terminal of mammalian and sea urchin PDI (23). The C-terminal of PDI ends with a KDEL sequence characteristic of ER-resident proteins (24). However, PDI has also been detected on the cell surface by immunocytology (25) and other techniques (26), in different cell types (27, 28) and platelets (29). Mammalian PDI has been regarded for many years to behave as a homodimer on gel filtration (30, 31) and to form a tetramer under certain conditions (32). However, recent reports suggest that it exists as a monomer under standard conditions (19, 33). Several properties of mammalian PDI domains have been studied. Recent investigations on recombinant fragments have elucidated domains involved in folding (18, 19, 34), catalysis (19, 34–36), chaperone activity (35, 37), and peptide/protein recognition (38).

A purified microsomal protein from sea urchin eggs binds 23 mol of Ca2+ at low affinity (20), shares certain characteristics with calsequestrin (39), is localized within the ER (40), has 55% identity with mammalian PDI and has PDI activity (23). Hence, we designated it ERcalcistorin/protein-disulfide isomerase (ECaSt/PDI) alluding to its putative dual functions within the ER (23). We subsequently showed that mammalian PDI binds 19 mol of Ca2+/mol of protein at low affinity (half-saturation) (12–14) or anti-chaperone activity (13). PDI has also been identified as a structural subunit of other enzymes, e.g. prolyl-4-hydroxylase (15) and the triglyceride transfer complex (16).

On the basis of sequence homology in the primary structure of PDI, Edman et al. (17) identified multiple domains, a, b, b’ and a’. The a domain (at the N-terminal) and the a’ (a portion of the C-terminal) are homologous with thioredoxin in their sequence and folded structure and each contains the CGHC sequence, the active sites. b and b’ are two homologous central domains with folded structures similar to thioredoxin but with no thioredoxin sequence homology (18). Interestingly, domain b shares a limited sequence identity with calsequestrin (19). These four domains therefore resemble thioredoxin folded structures (18). A domain, between the a domain and b domain was designated e because of its homology to the ligand-binding site of the estrogen receptor (11). Whether this should be regarded as a separate domain is now being questioned (19).
ration values, derived from the Hill equation were 2.77, 4.73, and 5.20 mM in the presence of 20, 100, or 100 mM KCl plus 3 mM MgCl₂, respectively (21). Thus, PDI presumably acts as a low affinity Ca²⁺ storage protein within the ER of mammals and sea urchins. We have recently obtained direct supporting evidence for such a role of ECaSt/PDI in the ER of a living cell (41). Hence PDI besides calreticulin (42) may be another major Ca²⁺ storage protein in the ER.

Suggestions that other ER proteins may serve a Ca²⁺ storage role have been based on their quantitation as high capacity low affinity calcium-binding proteins, e.g. CaBP2, previously designated ERp72 and characterized as containing three thioe-doxin-like active site domains (43). CaBP2 binds 12 mol of Ca²⁺/mol of protein, CaBP4 binds 11 mol of Ca²⁺/mol of protein (44), and endoplasmic 8–10 mol of Ca²⁺/mol of protein (45). CaBP4 (44) shows identity with GRP94 (46) and endoplasmin (45). Increased Ca²⁺ storage is also based on increased Ca²⁺ release by cells transfected with a putative Ca²⁺ storage protein, e.g. BIP (47).

The approximate correspondence of the number of calcium ions bound by ECaSt/PDI (20), mammalian PDI (21), and calreticulin (48) with the number of paired acidic residues in the respective molecules, led us to postulate, as already mentioned, that pairs of acidic residues were low affinity Ca²⁺-binding sites, but not exclusively, since Ca²⁺ could bind to single carboxyl group bridged to other groups (23). In support of our hypothesis we found, in this investigation, a correspondence between the percentage loss of paired acidic residues in the C-terminal truncated mutant and the percentage reduction in its Ca²⁺ binding capacity.

One might reasonably consider whether the abundant low affinity Ca²⁺ binding might also influence the enzyme activity of PDI. Hence, we studied the effect of Ca²⁺ on PDI activity using concentrations within the range and somewhat above the Kₐ values for Ca²⁺ binding which vary depending on ionic conditions. The choice of such concentrations was based on the assumptions that the free Ca²⁺ concentration within the inti-
mate microenvironment of ECaSt/PDI in the ER would be in equilibrium with the Kₐ values and that the published ER concentrations of free Ca²⁺ in the micromolar range (for re-
view, see Ref. 49), would not necessarily reflect the concentrations in the microenvironment of ECaSt/PDI or might not be accurate. Free Ca²⁺ concentrations in the ER up to 400 μM have been recently detected in cells transfected with a unique construct of green fluorescent protein fused with calmodulin (50). A considerably higher concentration of about 2 mM was considered to exist using Sr²⁺ as a surrogate for Ca²⁺ in cells transfected with an aequorin-Bip chimera (51). In any event, there are difficulties in obtaining accurate measurements of free Ca²⁺ in the ER (49, 52). The latter finding of an average Ca²⁺ concentration of about 2 mM suggests the possibility that the concentration may well be in the range of the Kₐ values for Ca²⁺ binding by ER Ca²⁺ storage proteins.

We now report that whereas 1 mM Ca²⁺ had a negligible effect on the enzyme activity of either ECaSt/PDI or the C-terminal truncated mutant using rRNase or insulin as sub-
strates, 5 mM Ca²⁺ had a moderate stimulating effect. How-
ever, with respect to the reactivation of reduced BPTI, 1–5 mM Ca²⁺ augmented the enzyme activity of ECaSt/PDI and the C-terminal truncated mutant. 5 mM Ca²⁺ also augmented this reactivation catalyzed by mammalian PDI. Surprisingly, ECaSt/PDI, in contrast to the C-terminal truncated mutant and mammalian PDI, prevented the spontaneous reactivation of rBPTI in the absence of Ca²⁺ suggesting that the C-terminal of ECaSt/PDI, differs from that of mammalian PDI, and has a charge distribution which enable it to complex with rBPTI and lock it in the reduced state in the absence of Ca²⁺.

**EXPERIMENTAL PROCEDURES**

**Materials**—Polylysine (molecular mass range 30–70 kDa), polyglutatoimic acid (31.4 kDa), poly(Glu,Ala,Tyr) (63:1, 50 kDa), and poly(Glu, Tyr) (1:1, 39 kDa) were supplied by Sigma. The Superose 12 column was supplied by Pharmacia. Iodination of reduced BPTI was performed using JODO-BEADS supplied by Pierce.

**Construction of cDNAs—**Clone Sa 1a1 of CaBP4 in pBluescript encoding ECaSt/PDI (23) was amplified by the polymerase chain reaction using a sense primer in combination with reverse primers to produce the complete molecule and the C-terminal truncated (Δ33–378) mutant. The forward primer 1016F (5'-ATATTTCTAGACATTTGCGGACGATTGCTGAAACTCAAGAAGATGTGTC-3') was designed to hybridize with the first 33 nucleotides encoding the mature protein at the 5' terminus of the ECaSt/PDI cDNA and contains a NdeI site (in bold), a stop codon (italics), and the sequence encoding the KDEL retention signal (underlined).

Polymerase chain reaction amplification was carried out using AmPIT transformed aq kit (Perkin-Elmer polymerase). Polymerase chain re-
action products were ligated into pCRII/AT cloning vector and Eshch
erichia coli HB101 was transformed with the ligation products as described (Invitrogen protocol). The fidelity of the sequence of the polymerase chain reaction products was confirmed by DNA sequencing (53). The complete cDNA was ligated into the NdeI/XhoI sites of the E. coli expression vector pARE4 and the truncated gene into the NdeI/ KpnI sites of the vector (54). Ligation products were used to transform BL21DE3 pLysS (E. coli) cells, a protease-deficient strain expressing T7 DNA polymerase under control of the isopropyl-1-thio-β-D-galactopy-
ranoside inducible promoter. Cells were grown at 37 °C in LB broth supplemented with 50 μg/ml ampicillin and 0.1 mM IPTG for 3–4 h. The culture was harvested at 600 nm reached 0.4. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and cells were grown for an additional 3 h.

**Purification of Recombinant ECaSt/PDI and the Mutant—**The purifica-
tion was according to the protocol described for the purification of recombinant human PDI (55) with some modifications. E. coli cells, expressing ECaSt/PDI and the mutant in 250 ml of culture, were collected by centrifugation at 5,000 × g for 15 min, resuspended in 50 ml of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 30 μM leupeptin. Cells were disrupted by sonication and the lysate spun down at 20,000 × g for 30 min. The supernatant was dialyzed 3 times against 4 liters of 25 mM sodium phosphate (pH 6.3). Partial purification was achieved by ion exchange chromatography. The dialysate was applied to a 2.5 × 35-cm column of DEAE-Sepharose equilibrated with the same buffer. The column was washed with 300 ml of 25 mM sodium phosphate (pH 6.3) and proteins were eluted by a linear gradient of 0 to 0.7 M NaCl (200 ml each) in 25 mM phosphate buffer (pH 6.3). Fractions (4 ml) containing PDI activity that eluted at approximately 0.37–0.45 M NaCl were analyzed by SDS-PAGE, combined, concentrated to 2–2.5 mg/ml by ultrafiltration, dialyzed against 100 volumes of 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA and stored frozen at −20 °C. Further purification was achieved by gel filtration in a Superose 12 column equilibrated and eluted in the same buffer. Samples (100 μl) were loaded onto the column and 0.5-ml fractions were collected at a flow rate of 0.5 ml/min. Protein elution was monitored by the absorbance at 280 nm and the eluate with the major absorbance peak and PDI activity was concentrated to 1.5–2 mg/ml by ultrafiltration and stored at −20 °C.

**Purification of ECaSt/PDI from Sea Urchin Eggs and Rabbit PDI from Liver**—ECaSt/PDI from sea urchin eggs (39) and mammalian PDI from rabbit liver (56) were purified as described.

**Calcium Binding**—The E. coli expressed ECaSt/PDI and the mutant (0.5–0.6 mg/ml) were purified and calcium binding was measured by equilibrium dialysis essentially as described (20). Briefly, protein samples (150 μl) were dialyzed in a Spectrofem semimicrodialysis tubing (4 mm, 12–14 kDa cut-off) against a buffer solution (20 ml) containing 20 mM MOPS (pH 7.0), 3 mM MgCl₂, and 50 or 150 mM KCl, and various concentrations of CaCl₂ with specific radioactivity of 250–300 cpm/mmol, at 4 °C for 24 h in capped polyethylene scintillation vials. At equilibrium, quadruplicate samples (30 μl) were taken from inside and
outside the dialysis tubing to determine radioactivity and duplicate samples (15 μl) from inside the dialysis tubing were taken to measure protein concentration (57). Calcium binding for each ECaSt concentration was determined in three experiments.

Reduction and Denaturation of BPTI—BPTI was reduced and denatured essentially as described (58). BPTI (4 mg) was suspended in a medium (200 μl) containing 8 mM guanidinium-HCl, 150 mM dithiothreitol, 200 mM Tris-HCl (pH 8.5), and stirred for 24 h at room temperature. The reduced, denatured protein was isolated by gel filtration on a Sephadex G-25 column equilibrated and eluted in 10 mM HCl. The presence of reduced BPTI in the column fractions (0.5 ml) was detected using 5,5′-dithiobis(2-nitrobenzoic acid) as described (59). Fractions containing rBPTI (1.5–2.0 mg/ml) were pooled and kept frozen at −20 °C until used. Under these conditions the reduced, denatured inhibitor was stable for at least 3 months. BPTI protein concentration was estimated spectrophotometrically at 280 nm (60).

Reactivation of rBPTI—The reactivation of rBPTI was assayed by monitoring its stoichiometric inhibitory effect on trypsin activity (58). PDI catalysis on refolding of BPTI occurs even in the absence of thiol redox buffer (61). Since we found the rate of spontaneous reactivation in the presence or absence of 5 mM CaCl₂ were loaded on a Superose 12,000 column (Pharmacia) that was equilibrated and eluted (0.5 ml/min) with the presence or absence of 5 mM CaCl₂ were loaded on a Superose 12,000 column (Pharmacia) that was equilibrated and eluted (0.5 ml/min) with the corresponding buffer. Fractions were collected at 0.5-min intervals and absorbance at 280 nm and radioactivity were monitored.

Nondenaturing Gel Electrophoresis—Purified ECaSt/PDI and the ECaSt/PDI-125I-rBPTI complex, isolated by gel filtration under conditions described above, were electrophoresed under nondenaturing conditions as described for the electrophoresis of acidic proteins using a high pH discontinuous buffer system (63). The lane containing purified ECaSt/PDI was cut from the gel and stained with Coomassie Blue. The presence of the ECaSt/PDI,125I-rBPTI complex in other lanes of the gel was detected immediately after electrophoresis by exposing the wet, unstained gel to x-ray film (Kodak X-Omat) for 24 h at 4 °C.

Acid Gel Electrophoresis—ECaSt/PDI (20 μg) and rBPTI (7 μg) were mixed in 20 μl of buffer containing 100 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA at 22 °C for 30 min. The mixture was then subjected to acidic gel electrophoresis under nondenaturing conditions as described (64).

RESULTS

Depicted in Fig. 1 are the six mammalian PDI domains (4) placed in corresponding positions in the block diagrams of the primary structures of ECaSt/PDI and its C-terminal truncated mutant. The boundaries of the domains were assigned on the basis of sequence homology. The domains a and a’ of ECaSt/PDI and the C-terminal truncated mutant were 32% identical, share 59 and 62% identity with the corresponding domains in mammalian PDI and each one contains the thioredoxin-like active site (CGHC). The domains b and b’ in ECaSt/PDI are 20% identical and share 50 and 51% identity with the corresponding domains in mammalian PDI. The e domain is 50% identical to the e domain of mammalian PDI and 20% identical to the human estrogen receptor. The c domain of ECaSt/PDI contains the last 29 residues (451–479) of the C-terminal to correspond with the 29 residues (463–491) of the c domain of human PDI (18) and they have a 28% identity. The chosen human c domain originally contained 43 residues (4). Interestingly, the more recent changed number of residues (463–491) (18, 35) in the human e domain we had previously designated the “tail end” of its C-terminal (23). The C-terminal truncated mutant (Δ385–475) lacks part of the a’ and the c domain. It was designed to retain the thioredoxin active site at residues 374–377 and to truncate the cluster of paired acidic residues in the C-terminal.

Apparent Molecular Weights of Recombinant ECaSt/PDI and the C-terminal Truncated Mutant—Recombinant ECaSt/PDI and the mutant were produced in E. coli with yields and purity similar to those reported for recombinant human PDI (55). Full-length ECaSt/PDI and the C-terminal truncated mutant migrated at 58 and 45 kDa, respectively, on SDS-PAGE (Fig. 2) and are close to the 53- and 43-kDa values deduced from the amino acid content of the respective molecules.

Calcium Binding Properties of Recombinant ECaSt/PDI and the C-terminal Truncated Mutant—In previous studies ECaSt/PDI isolated from the sea urchin egg bound 23 mol of Ca2+/mol of protein. The half-binding maxima determined from the Hill equation were 1.6, 3.63, 4.81, and 5.73 mM in the presence of 20 mM KCl, 100 mM KCl, 100 mM KCl plus 3 mM MgCl₂, and 250 mM KCl plus 3 mM MgCl₂, respectively (20). Similarly, the half-binding maxima of Ca2⁺ to mammalian PDI increased with increasing ionic strength of the media (21); the values are given under “Introduction.” The Ca2⁺ binding properties of recombinant ECaSt/PDI and the C-terminal truncated mutant

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**Fig. 1.** PDI domains represented in ECaSt/PDI and its deletion mutant (Δ385–475). Above the dotted line indicate the extent of the deletion made to produce the mutant. Numbers under each block indicate the extent of each domain in the primary sequence. Dashed segments within a and a’ domains indicate the thioredoxin-like active sites (CGHC). The end segments of the C-terminal represent the KDEL retention signals.
were studied in the medium described for native ECaSt/PDI (20) at two different concentrations of KCl. At 50 mM recombinant ECaSt/PDI bound 26 mol of Ca\(^{2+}\)/mol of protein with a \(K_d\) of 2.8 mM (Fig. 3A). The truncated mutant bound 14 mol of Ca\(^{2+}\)/mol of protein with a \(K_d\) of 2.8 mM (Fig. 3A). At 150 mM KCl the \(K_d\) for Ca\(^{2+}\) binding was 5.2 mM for both molecules while the maximal Ca\(^{2+}\) binding was 25.6 and 14.2 mol of Ca\(^{2+}\)/mol of protein for ECaSt/PDI and the truncated mutant, respectively (Fig. 3B). Therefore the C-terminal truncated ECaSt/PDI showed a 48% reduction in the binding capacity which interestingly corresponds to a reduction of 45% of pairs of acidic residues, a reduction of 9 out of a total of 20 pairs in ECaSt/PDI. This is in keeping with our previous hypothesis that carboxyl pairs are the main low affinity Ca\(^{2+}\) binding.

Effect of Ca\(^{2+}\) on the PDI Isomerase Activity Using Insulin or Reduced RNase as Substrates—Under the conditions optimized for mammalian PDI (23), recombinant ECaSt/PDI and its C-terminal truncated mutant displayed similar specific activities on rRNase and insulin as substrates. The refolding of reduced rRNase and the reduction of insulin by GSH were catalyzed by both ECaSt/PDI and the mutant with specific activities of 0.21 mol of RNase renatured/min/\(\mu\)mol of protein and 0.2 \(\mu\)mol/min/mg of protein, respectively. Thus the deletion of the C-terminal, involving part of the \(\alpha\) domain and \(\epsilon\) domain, does not seem to affect ECaSt/PDI activity when insulin or rRNase are substrates. Those activities were augmented moderately, 1.8- and 2.2-fold, respectively, by 5 or 10 mM CaCl\(_2\) or 10 mM MgCl\(_2\) or polylysine (data not shown).

Effects of Ca\(^{2+}\) or Polylysine on the Reactivation of rBPTI by ECaSt/PDI, the C-terminal Truncated Mutant and Mammalian PDI—We studied the effects of ECaSt/PDI, its C-terminal truncated mutant, and mammalian PDI on reactivation of rBPTI in the absence and presence of Ca\(^{2+}\) or polylysine. Reactivation of rBPTI was assayed by monitoring the rate of recovery of its inhibitory activity on trypsin. The assay carried out in the absence of redox buffer resulted in a slow rate of spontaneous reactivation of rBPTI which displayed biphasic kinetics with a lag phase of about 60 min preceding a linear increment (Fig. 4, A-D). Fifty percent (\(t_{1/2}\)) of spontaneous reactivation of rBPTI occurred in 220 min and was insensitive to Ca\(^{2+}\) or polylysine (data not shown). This reaction was done at pH 7.4, slightly alkaline, to promote spontaneous disulfide bond formation and reactivation but at a rate slow enough to assess any influence of a possible activator such as Ca\(^{2+}\) (Fig. 4).

Surprisingly, recombinant ECaSt/PDI prevented this spontaneous reactivation observed over a period of 3 days in the absence of Ca\(^{2+}\) (Fig. 4A). Native ECaSt/PDI purified from the sea urchin eggs had a similar effect (data not shown). The addition of 5 mM CaCl\(_2\) (or 10 \(\mu\)M polylysine) to the solution prior to mixing of ECaSt/PDI with rBPTI accelerated the reactivation of rBPTI by ECaSt/PDI, the \(t_{1/2}\) was reduced from 220 to 85 min (Fig. 4A). The addition of 5 mM MgCl\(_2\) to the solution, prior to mixing of the two proteins, had no effect on the inhibition of rBPTI reactivation (Fig. 4, panel A). Neither 5 mM CaCl\(_2\) nor 10 \(\mu\)M polylysine reverted the inhibitory effect of ECaSt/PDI when added after both proteins were mixed (not shown).

The C-terminal truncated mutant, in contrast to full-length ECaSt/PDI, catalyzed the reactivation of reduced BPTI with a \(t_{1/2}\) of 149 min in the absence of CaCl\(_2\) (Fig. 4B). This reactivation was accelerated by 5 mM CaCl\(_2\) or 10 \(\mu\)M polylysine (\(t_{1/2}\) =...
80 min) and was insensitive to 5 mM MgCl₂ (Fig. 4 B). We confirmed the previous finding that mammalian PDI activated rBPTI in the absence of CaCl₂ (3, 61). Under our experimental conditions the reactivation showed a t₁/₂ of 63 min (Fig. 4, panel C) that was further reduced to 32 min (Fig. 4, panel C) by 5 mM CaCl₂. The reactivation of rBPTI by ECaSt/PDI, the mutant, and mammalian PDI are compared in Table I.

These experiments were also done at pH 7.0 in the presence of 150 mM KCl, in the same medium used for Ca²⁺ binding assay, and the effects of Ca²⁺ at 1, 2.5, and 5 mM were tested. As expected the spontaneous reactivation took place at a much reduced rate, too slow to determine the t₁/₂ (Fig. 5, A and B). However, the effects of ECaSt/PDI and the C-terminal truncated mutant were essentially similar to those described in Fig. 4. ECaSt/PDI prevented the spontaneous reactivation of rBPTI in the absence of Ca²⁺. A progressive acceleration of rBPTI reactivation was observed with increasing concentration of Ca²⁺. The t₁/₂ for reactivation were 240, 170, and 87 min in the presence of 1, 2.5, and 5 mM Ca²⁺, respectively (Fig. 5 A). Thus, 5 mM Ca²⁺ further augmented the activation by 1.5 mM Ca²⁺ 2.8-fold. The C-terminal truncated mutant reactivated rBPTI with t₁/₂ of 125 min in the absence of Ca²⁺. The reactivation increased progressively with t₁/₂ of 110, 90, and 75 min in the presence of 1, 2.5, and 5 mM Ca²⁺, respectively (Fig. 5B).

Thus 1–5 mM Ca²⁺ augments the reactivation of rBPTI by ECaSt/PDI, by its C-terminal truncated mutant and by mammalian PDI. The C-terminal truncated ECaSt/PDI behaves essentially like mammalian PDI. Both molecules enhance the reactivation of rBPTI in the absence of Ca²⁺ and Ca²⁺ further augments the reactivation. It appears therefore that the C-terminal portion of ECaSt/PDI differs from that of mammalian PDI and somehow allows for complex formation of the molecule with rBPTI in the absence of Ca²⁺, a state which prevents the spontaneous refolding of rBPTI. Once the complex is formed Ca²⁺ or polylysine cannot dissociate the molecules. Such a complex did form and is described below.

![Fig. 4. Kinetics of reactivation of reduced BPTI.](image) Native BPTI was reduced and subsequently reactivated as described under “Experimental Procedures.” Panel A shows the spontaneous reactivation of rBPTI (○) which was not affected by the addition of 5 mM CaCl₂, 5 mM MgCl₂, or 10 μM polylysine (not shown). Spontaneous reactivation of rBPTI was blocked by ECaSt/PDI (△) which was not influenced by 5 mM MgCl₂ (▲). Addition of 5 mM CaCl₂ (●) or 10 μM polylysine (□) to full-length ECaSt/PDI prior to mixing with rBPTI resulted in marked augmentation of the reactivation of rBPTI. Panel B shows spontaneous reactivation of rBPTI (○) which was increased by the C-terminal truncated mutant (△) with no difference in the presence of 5 mM MgCl₂ (▲). Addition of 5 mM CaCl₂ (●) or 10 μM polylysine (□) augmented the activity by the mutant. Panel C, mammalian PDI (△) accelerated the spontaneous reactivation of rBPTI (○) which was further augmented by the addition of 5 mM CaCl₂ (●). Panel D, the spontaneous reactivation of rBPTI (○) was blocked by 10 μM polyglutamate (△). Each value is the mean of two independent experiments.

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TABLE I

Comparison of effects of mammalian PDI, ECaSt/PDI, and the C-terminal truncated mutant on the reactivation of rBPTI at pH 7.4

| t₁/₂ of rBPTI reactivation | Degree of activation |
|----------------------------|---------------------|
| Spontaneous reactivation   | 220                 |
| + Mammalian PDI + 5 mM Ca²⁺| 32                  | 6.9×                |
| + ECaSt/PDI + 5 mM Ca²⁺    | 85                  | 2.6×                |
| + Mammalian PDI – Ca²⁺     | 63                  | 3.5×                |
| + mutant – Ca²⁺            | 149                 | 1.5×                |
| + Mammalian PDI + 5 mM Ca²⁺| 32                  | 6.9×                |
| + mutant + 5 mM Ca²⁺       | 80                  | 2.8×                |

Each value is the mean of two independent experiments.

The t₁/₂ of rBPTI reactivation by mammalian PDI, ECaSt/PDI, and the mutant determined in Fig. 4 are compared in terms of their degree of activation.
polyglutamates mimicked the behavior of ECaSt/PDI. Only the effect of the homopolymer polyglutamate is presented (Fig. 4, panel D).

Isolation of an ECaSt/PDI–125I-rBPTI Complex by Gel Filtration—The prevention of rBPTI reactivation by ECaSt/PDI (Fig. 6A) in the absence of Ca\(^{2+}\) suggested the formation of a complex between the two molecules. To study this possibility \(\text{125I-rBPTI}\) and ECaSt/PDI were mixed in the presence and absence of 5 mM CaCl\(_2\) and the mixtures were analyzed by gel filtration chromatography. Fig. 6A shows the elution peak of ECaSt/PDI detected by absorbance at 280 nm. Fig. 6B shows the overlapping radioactive and absorbance peaks of \(\text{125I-rBPTI}\). A mixture of ECaSt/PDI and \(\text{125I-rBPTI}\) in the absence of Ca\(^{2+}\), eluted as a complex with overlapping absorbance and radioactive peaks, with a retention time close to that of ECaSt/PDI alone (Fig. 6C). When 5 mM CaCl\(_2\) was added to the incubation medium before ECaSt/PDI and \(\text{125I-rBPTI}\) were mixed, two distinct peaks of radioactivity were detected; a large radioactive peak that co-eluted with rBPTI and a small radioactive peak that co-eluted with ECaSt/PDI (Fig. 6D). Thus, in the absence of Ca\(^{2+}\), rBPTI and ECaSt/PDI form a complex that is stable during gel filtration and the presence of Ca\(^{2+}\) prevented the complex formation.

Such complex formation was confirmed by gel electrophoresis under nondenaturing conditions and by autoradiography. An elution sample containing the ECaSt/PDI–125I-rBPTI complex in the absence of Ca\(^{2+}\) (see peaks in Fig. 6C) migrated electrophoretically as a complex; the autoradiogram in Fig. 7A, lane 2, shows a single radioactive band that co-migrates with ECaSt/PDI in the absence of Ca\(^{2+}\) (lane 2 also shows that no detectable radioactive band was associated with the minor protein impurities traveling above ECaSt/PDI (lane 1)). On the other hand, a sample containing mainly ECaSt/PDI in the presence of Ca\(^{2+}\) (see peaks in Fig. 6D) shows no radioactive band (lane 3), thus confirming that Ca\(^{2+}\) dissociated the complex.

Redox State of BPTI in the Complex—To determine if rBPTI in the complex with ECaSt/PDI remained reduced or underwent some partial reoxidation, the complex was electrophoresed in an acidic nondenaturing gel which resolves reduced and partially or completely oxidized forms of BPTI (64). The acidity dissociates the complex and a single band was associated with the minor protein impurities traveling above ECaSt/PDI (lane 1). On the other hand, a sample containing mainly ECaSt/PDI in the presence of Ca\(^{2+}\) (see peaks in Fig. 6D) shows no radioactive band (lane 3), thus confirming that Ca\(^{2+}\) dissociated the complex.
apparently binds all 20 molecules of \( \text{Ca}^{2+} \) (48).

Calsequestrin does not have sufficient carboxylic pairs in its primary structure to account for the number of low affinity \( \text{Ca}^{2+} \) bound but has 110 acidic residues. If our hypothesis is correct, the necessary carboxyl pairs, or a single one in association with another group, would be formed in the folded molecule (23). The crystal structure of calsequestrin from rabbit skeletal muscle has now been determined and the authors describe the presence of three folded thioredoxin domains each having a hydrophobic core with acidic residues having 13–36 negative charges on the surface generating an electronegative potential (65). This together with clefts formed in the dimeric state constitute the sites for low affinity \( \text{Ca}^{2+} \) binding. The number of pairs of negatively charged groups that may have been formed in these configurations is not given (65). MacLennan and Reithmeier (66), on reviewing the significance of this article, succinctly place these and the other structural findings into the context of the function of the sarcoplasmic reticulum and the ER in intracellular \( \text{Ca}^{2+} \) regulation. With regard to calcium storage in relation to its release, calsequestrin is better understood than any of the calcium storage proteins in the ER. For example, information is evolving on its coupling to the ryanodine \( \text{Ca}^{2+} \) release channel involving conformational changes (67) and connections with the junctional membrane (68), triadin (69), and junctin (70).

On determining that both sea urchin and mammalian PDI could bind an abundant number of calcium ions, we supposed that \( \text{Ca}^{2+} \) would play a role in their isomerase activities at concentrations in the range of and above their \( K_d \) values for \( \text{Ca}^{2+} \) binding. And indeed, 5 mM \( \text{Ca}^{2+} \) augmented the activity of ECaSt/PDI and the C-terminal truncated mutant about 2-fold, a moderate degree, using RNase and insulin as substrates. Primm et al. (71) using the same concentration of \( \text{Ca}^{2+} \) and denatured RNase as a substrate, obtained a negligible effect on the activity of mammalian PDI. However, the chaperone and anti-chaperone activities of PDI were modulated by \( \text{Ca}^{2+} \) in the millimolar range of concentration, with a maximal effect at around 5 mM (71).

The effect of \( \text{Ca}^{2+} \) was more dramatic on the activity of ECaSt/PDI when rBPTI was the substrate (Fig. 4). In the absence of \( \text{Ca}^{2+} \), ECaSt/PDI prevented the spontaneous reactivation of rBPTI due to complex formation between the two molecules which was determined by gel filtration and electrophoresis under non-denaturing conditions. The presence of 5 mM \( \text{Ca}^{2+} \) prevented complex formation with ECaSt/PDI and resulted in a 2.6-fold augmentation of the spontaneous reactivation of rBPTI at pH 7.4. At pH 7.0, 1 mM \( \text{Ca}^{2+} \) reduced markedly the immeasurable \( t_{1/2} \) of spontaneous reactivation to 240 min which was further accelerated by increasing concentrations of \( \text{Ca}^{2+} \); 5 mM \( \text{Ca}^{2+} \) further augmented the reactiva-
polyglutamate on rBPTI (Fig. 4D). The apparent shielding of negative charges by polylysine at low concentrations (10 μM) suggests the possibility that ER proteins with a cluster of basic residues could shield negative charges in ECaSt/PDI resulting in a modulation of its interactions with other proteins. Unlike ECaSt/PDI, mammalian PDI catalyzes the refolding of rBPTI in the absence of Ca\(^{2+}\) (Fig. 4C, also see Refs. 3, 61, and 72) and others found that the deletion of its c domain had no effect on the catalysis of disulfide bond rearrangement in BPTI intermediates as substrates (36). Furthermore, a fragment of BPTI could not be cross-linked to the isolated α'-c domains of mammalian PDI in the absence of Ca\(^{2+}\), suggesting that these mammalian PDI domains do not interact strongly with BPTI (38). Hence, the C-terminals of ECaSt/PDI and mammalian PDI which have a 44% identity must differ in charge distributions and electrostatic potential on their surfaces even though they have a similar number of acidic residues (23). ECaSt/PDI and mammalian PDI also differed in the degree of isomerase activities. ECaSt/PDI has 30% of the activity of mammalian PDI on substrates rRNase and insulin (23) and in this investigation similarly augmented to a lesser extent the reactivation of rBPTI than did mammalian PDI (see Table I).

These in vitro results demonstrate a role of Ca\(^{2+}\) on mammalian PDI and ECaSt/PDI isomerase activity. In cells in culture, Ca\(^{2+}\) also appears to play a role in maintaining the structure of ER proteins. Lodish and co-workers (73) have shown, by the use of Ca\(^{2+}\) ionophores, an inhibition of the exit of secretory proteins, to different extents depending on the protein, probably due to a disruption of their folding associated with the depletion (or reduction) of Ca\(^{2+}\) in the ER. In another study, Ca\(^{2+}\) ionophores and thapsigargin impaired the maturation and proper disulfide bond of an expressed model protein, the H1 subunit of the asialoglycoprotein receptor, containing 8 cysteine residues in its exoplasmic domain (74). The authors speculate that the unfolded state of the H1 subunit, probably due to the reduced PDI activity in the absence (or in reduced concentration) of Ca\(^{2+}\), may be the cause preventing its exit from the ER. However, ionophores and thapsigargin had a marginal effect on secretion of albumin (73, 74), a protein containing many cysteine residues, 35 in human serum albumin. If the H1 subunit and albumin are substrates of PDI in vivo then their PDI-catalyzed folding differs in response to the decrease of Ca\(^{2+}\) concentration in the ER. Similarly, our in vitro results also show differences in Ca\(^{2+}\)-dependent responses of the substrates rRNase, insulin, and rBPTI. Furthermore, mammalian PDI and ECaSt/PDI respond differently to Ca\(^{2+}\) for a given protein substrate. The depletion (or reduction) of Ca\(^{2+}\) in the ER also facilitates the dissociation of variants of another expressed protein, the T-cell antigen receptor α chain from the immunoglobulin heavy chain binding protein (BiP) (75). In summary, proper folding (73), including disulfide bond formation (74) and protein–protein interactions (75) in the secretory pathway of the cell seem to be effected by the Ca\(^{2+}\) content in the ER and interestingly, different secretory proteins are affected differently.

Our in vitro experiments show that the effect of Ca\(^{2+}\), in the millimolar range of concentrations, on ECaSt/PDI activity was dependent on the protein substrate. With insulin or rRNase the activity was insensitive to 1 mM Ca\(^{2+}\) and moderately stimulated at 5 mM Ca\(^{2+}\). However, ECaSt/PDI activity on rBPTI was absolutely dependent on Ca\(^{2+}\) which produced a substantial effect at 1 mM concentration, by dissociating ECaSt/PDI from rBPTI and then accelerating the activation of ECaSt/PDI. Our studies also show activation of mammalian PDI by 5 mM Ca\(^{2+}\).
Whether the modulations of Ca\(^{2+}\) concentrations around the K\(_T\) values for Ca\(^{2+}\) binding to PDI play a role in regulating PDI activity in the ER of a cell remains to be determined. Also worthy of further considerations is whether Ca\(^{2+}\) affects any particular substrate directly.

The continuing studies on the domains of mammalian PDI and ECaSt/PDI and the possible future elucidation of their tertiary structures will lead to a better understanding of their roles as isomerases and Ca\(^{2+}\) storage proteins and the relationship between these two functions within the endoplasmic reticulum.

REFERENCES

1. Epstein, C. F., Goldeberger, R. F., and Anfinsen, C. B. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 439–449
2. Venetiyaner, P., and Straub, F. B. (1963) Biochim. Biophys. Acta 76, 166–168
3. Creighton, T. E., Hillson, D. A., and Freedman, R. B. (1980) J. Mol. Biol. 142, 251–263
4. Freedman, R. B., Hirsh, T. R., and Tuite, M. F. (1994) Trends Biochem. Sci. 19, 331–336
5. Lu, J. M., and Lennarz, W. J. (1996) Exp. Suppl. (Basel) 77, 97–117
6. Gilbert, H. F. (1997) J. Biol. Chem. 272, 29399–29402
7. Noiva, R., and Lennarz, W. J. (1992) J. Biol. Chem. 267, 3553–3556
8. Wells, W. W., Xu, D. P., Yang, Y., and Rocque, F. A. (1990) J. Biol. Chem. 265, 15361–15364
9. Cheng, S., Gong, Q., Parkinson, C., Robinson, E. A., Appella, E., Merlino, G. T., and Pastan, I. (1987) J. Biol. Chem. 262, 11221–11227
10. Guthapfel, R., Gueguen, P., and Quemeneur, E. (1996) Eur. J. Biochem. 242, 313–319
11. Tsibris, J. C. M., Hunt, L. T., Ballejo, G., Barker, W. C., Toney, L. J., and Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) J. Biol. Chem. 260, 1744–1749
12. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllyla, R., Huhtala, L., Koivu, Y. (1990) J. Biol. Chem. 265, 272, 365, 563–577
13. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) Nature 317, 267–270
14. Kassell, B. (1970) Methods Enzymol. 28, 6764–6771
15. Venetianer, P., and Straub, F. B. (1963) Biochim. Biophys. Acta 76, 166–168
16. Creighton, T. D. (1974) Anal. Biochem. 58, 72, 217–232
17. Ellman, G. L. (1959) Arch. Biochem. Biophys. 72, 4112–4116
18. Freedman, R. B., Hirst, T. R., and Tuite, M. F. (1994) Trends Biochem. Sci. 19, 331–336
19. Lu, J. M., and Lennarz, W. J. (1996) Exp. Suppl. (Basel) 77, 97–117
20. Lebeche, D., and Kaminer, B. (1992) J. Biol. Chem. 267, 25752–25756