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CALCIUM BINDING PROPERTIES OF RABBIT LIVER PROTEIN DISULFIDE ISOMERASE

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Summary: We have characterized the Ca^{2+} binding properties of protein disulfide isomerase (PDI). It binds 19 mol Ca^{2+}/mol protein with low affinity, which is reduced by increasing the ionic strength. Ca^{2+} induced conformational changes detected by UV difference spectroscopy. These Ca^{2+} binding properties resemble those of a sea urchin egg 58 kDa protein.

The endoplasmic reticulum (ER), a multifunctional organelle, plays an important role in regulating the cytoplasmic Ca^{2+} concentration by actively taking up Ca^{2+}, storing it and releasing it on receiving the appropriate signal (see review in 1). A major ER calcium storage protein with high calcium binding capacity and low affinity is calreticulin, a protein functionally analogous to calsequestrin in the sarcoplasmic reticulum of striated muscle (see review in 2).

We identified, purified and characterized a 58 kDa calsequestrin-like protein from the ER of sea urchin eggs (3) and subsequently found it to be a high capacity (23 mol Ca^{2+}/mol protein), low affinity Ca^{2+} binding protein distinct from calsequestrin and calreticulin (4). On sequencing the gene encoding for this distinct protein we found it to have a 55% sequence identity with mammalian protein disulfide isomerase (PDI) and a 67% similarity on conservative amino acid substitution. It had 30% of the enzyme activity of rabbit liver PDI (manuscript submitted). These findings prompted us to determine the Ca^{2+} binding properties of mammalian PDI, an ER resident protein. In the present communication we report on the quantitation of Ca^{2+} binding by PDI and the associated UV spectral changes, which resemble those of the sea urchin egg 58 kDa protein.

METHODS

Purification of PDI: Protein disulfide isomerase was purified from rabbit liver according to Hillson et al. (5). Final purified fractions eluted through a DEAE-Sephacel column were pooled, dialyzed against 50 mM ammonium bicarbonate, lyophilized and stored at -20° C. Protein concentrations were determined by the Bradford method (6). SDS polyacrylamide gel electrophoresis was done according to Laemmli (7).

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Equilibrium Dialysis : $^{45}Ca^{2+}$ binding by PDI was determined by equilibrium dialysis as described (4). Briefly, protein samples (0.7 mg/ml) were decalcified by dialyzing against an EGTA-containing buffer (20 mM Mops, pH 7.0, 1 mM EGTA and 20-100 mM KCl) for 36 h at 4° C and then against the same buffer without EGTA. Thereafter, the protein samples were dialyzed for 24 h at 4° C against 20 ml of 20 mM Mops, pH 7.0, 20-100 mM KCl, plus or minus 3 mM $MgCl_2$ and various concentrations of $CaCl_2$ (containing $^{45}Ca^{2+}$ at a specific activity of 10^6 cpm/mmole). Duplicate 30-ml aliquots were taken from the sample solutions and the dialysates and radioactivity was measured by scintillation counting (Packard Tri-Carb). Ca^{2+} binding curves were determined by a non-linear least squares fitting procedure using the program Asystant (Asyst Software Technologies, Inc.) on an AST PC. Protein determination was performed on samples, in duplicate, using the Bradford method (6).

Spectroscopic Analyses: Conformational changes induced by Ca^{2+} binding to PDI were studied by UV difference spectroscopy as described (4).

RESULTS

Ca^{2+} binding measurements:

The purified PDI (see Fig. 1) bound an average of 340 nmol Ca^{2+}/mg of protein at saturation (Fig. 2), corresponding to about 19 mol Ca^{2+}/mol protein based on the monomeric molecular mass of 57 kDa (8). The half-saturation values, derived from the Hill equation (9), were 2.77 mM, 4.73 mM and 5.20 mM in the presence of 20 mM KCl, 100 mM-KCl and 100 mM KCl plus 3 mM $MgCl_2$, respectively. Evidently, the affinity of PDI for Ca^{2+} was reduced by the increase in ionic strength, with only a small influence by Mg^{2+} (Fig. 2). Hill coefficients under the above conditions were 1.56, 1.53 and 1.68 respectively. These values, being slightly higher than unity, possibly indicate a mild cooperativity of calcium binding.

Ca^{2+}-induced spectral changes:

The UV difference spectrum between samples of PDI containing Ca^{2+} and those free of Ca^{2+} is depicted in Fig. 3A. The absorption was slightly reduced at 282 nm (the maximum absorption

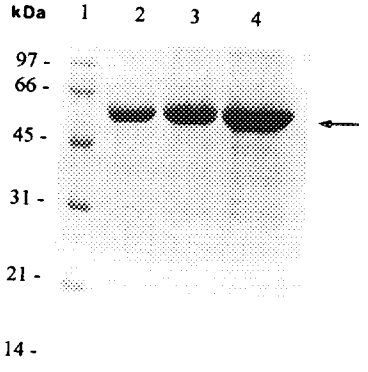

Fig. 1. **Polyacrylamide gel electrophoresis of PDI.**
Increasing concentrations of PDI were run on a 15% SDS gel and stained with Coomassie Blue. Lane 1, MW standards (kDa); lane 2, 4.25 µg; lane 3, 8.5 µg; lane 4, 17 µg. *Arrow* indicates PDI. Minor impurity bands can be seen.

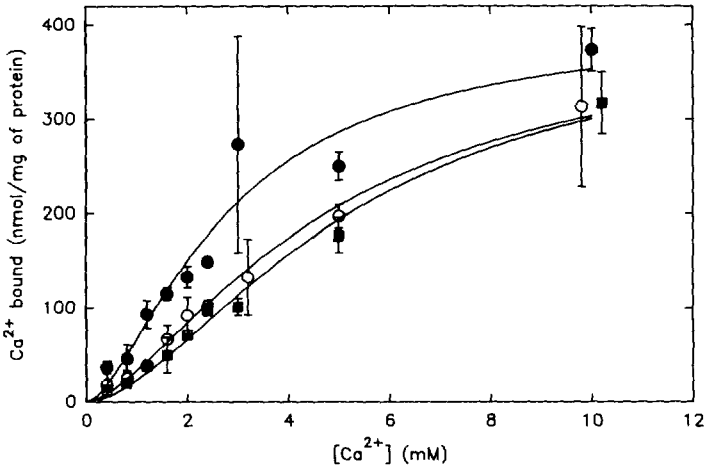

Fig. 2. **Ca^{2+}-binding by PDI.**
Ca^{2+}-binding of PDI was determined by equilibrium dialysis. Protein samples were dialyzed for 24 h at 4° C against 20 ml of a buffer containing 20 mM Mops (pH 7.0) and 20 mM KCl (●), 100 mM KCl (○) and 100 mM KCl plus 3 mM $MgCl_2$ (■). Each data point represents the mean ± S.E.M. of 6 determinations.

peak). A progressive decrease in absorption at 282 nm occurs with increasing concentrations (1-10 mM) of Ca^{2+} (Fig. 3B). These Ca^{2+}-induced UV absorption changes are similar to those observed in the 58 kDa protein from the sea urchin eggs (4). An approximate *Kd* value of 4 mM was estimated from the concentrations producing a half-maximal change, a value close to the half-saturation value of 4.73 mM from the binding studies under similar ionic conditions (100 mM KCl - see above). There were no significant intrinsic fluorescence changes in the presence of Ca^{2+}.

DISCUSSION

PDI is one of a number of resident proteins in the ER which play an important role in co- and post-translational modifications of nascent polypeptides. As first shown in Anfinsen's laboratory, it catalyzes the appropriate formation of S-S bonds in proteins (10). Besides this function, leading to proper folding of SH containing proteins (11), PDI has been implicated in several other activities (see review in 12). It catalyzes the GSH-dependent reduction of dehydro-L-ascorbate (13), is involved in peptidyl proline hydroxylation (14) and in transfer of triglyceride and cholesteryl ester across the ER membranes (15). It binds triiodothyronine (16) and other peptides (17,18). Consideration is being given to its chaperone properties (19,20,21).

PDI was found to bind $^{45}Ca^{2+}$ using the overlay technique but no quantitative values were obtained (22). We now present the first quantitative study of Ca^{2+} binding by this enzyme. The average maximum binding of 19 mol of Ca^{2+}/mol of PDI (with low affinity) (Fig. 2), approximates the 23 moles of Ca^{2+} bound per mole of the previously characterized 58 kDa egg

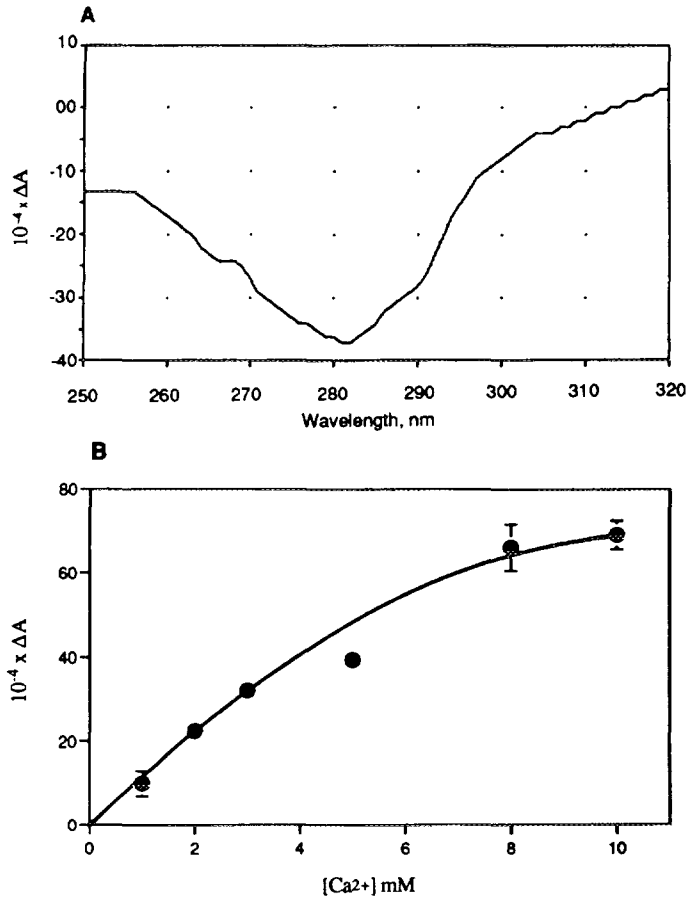

Fig. 3. **UV absorption difference spectra of PDI induced by Ca^{2+}.** UV absorption difference spectra of PDI were measured between the sample cuvette containing varying concentrations of Ca^{2+} and the reference cuvette containing no Ca^{2+}. 1mg/ml of protein was dissolved in 1 ml of 20 mM Mops (pH 7.0), 100 mM KCl and 0.1 mM EGTA. A) Effect of 5 mM $CaCl_2$. The absorption is reduced maximally at 282 nm. B) The changes in the absorption at 282 nm are expressed as a function of increasing free Ca^{2+} concentrations (1-10 mM). *Bars* represent the S.E. of 3 determinations.

protein (4). These relatively high Ca^{2+} capacities are consistent with the acidic nature of both molecules.

The changes in the UV absorption spectra reflect changes in the environment of the aromatic residues associated with Ca^{2+}-induced conformational changes of PDI. The enzyme contains a considerable number of aromatic residues, 32 phenylalanines, 12 tyrosines and 5 tryptophans (16). Hence, the spectral measurements are indicative of an overall average change of the specific hydrophobic and hydrophilic environments of the aromatic residues. A detailed interpretation will only be possible when the tertiary structure of the molecule is known.

The high Ca^{2+} binding capacity of PDI suggests that it may have Ca^{2+} storage and buffering functions in the ER. On comparing the Ca^{2+} binding capacities of a number of ER

Table 1

Ca^{2+} binding parameters of high capacity, low affinity ER Ca^{2+} binding proteins. Ca binding was determined by equilibrium dialysis or by a $^{45}Ca^{2+}$ overlay technique in the studies of endoplasmin.

Protein	K_d (mM)	B_{max} (mol/mol)	Ref.
PDI	4.7	19	this study
egg 58 kDa	3.5	23	4
Calreticulin	1.0	25	2
Endoplasmin	6.0	8-10	30
CaBP2	0.4	12	31
CaBP4	0.6	11	31

resident proteins, we found that calreticulin, the 58 kDa sea urchin protein and PDI bound about twice the number of Ca^{2+}/mole of protein compared to endoplasmin, CaBP2 and CaBP4 (Table 1). These ER proteins and possibly others, probably act as Ca^{2+} buffers in the ER. The calcium content within the ER, determined by electron probe x-ray micro-analysis is about 5 mM and may be more, depending on conditions (23,24). The concentration of calcium in the ER is known to influence ER proteins in a number of ways. The reduction of ER calcium affects protein folding (25), secretion out of the ER (26,27,28), and the binding and release of ER proteins by the immunoglobulin heavy chain binding protein (BiP) within the ER (29).

In addition to their Ca^{2+} buffering role, PDI and other ER resident proteins, may require an optimal number of Ca^{2+} bound to them to carry out their respective functions efficiently, particularly with respect to their role in the maturing and chaperoning of non-resident ER proteins.

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REFERENCES

1. Berridge, M.J. (1993) Nature **361**, 315-325
2. Michalak, M., Milner, R.E., Burns, K. and Opas, M. (1992) Biochem. J.**285**, 681-692
3. Oberdorf, J., Lebeche, D., Head, F.J. and Kaminer, B.(1988) J. Biol. Chem. **263**, 6806-6809
4. Lebeche, D. and Kaminer, B. (1992) Biochem. J., **287**, 741-747
5. Hillson, D.A., Lambert, N. and Freedman, R.B. (1984) Methods Enzymol **107**, 281-294
6. Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254
7. Laemmli, U.K. (1970) Nature **277**, 680-685
8. Edman, J.C., Ellis, L., Blacher, R.W., Roth, R.A. and Ritter, W.J. (1985) Nature **317**, 267-270
9. Hill, A.V. (1910) J. Physiol. (London) **40**, iv-vii
10. Goldberger, R.F., Epstein, C.F. and Anfinsen, C.B. (1963) J. Biol. Chem., **238**, 628-635
11. Creighton, T.E., Hillson, D.A. and Freedman, R.B. (1980) J. Mol. Biol. **142**, 43-62
12. Noiva, R. and Lennarz, W.J. (1992) J. Biol. Chem., **267**(6), 3553-3556

13. Wells, W.W., Xu, D.P., Yang, Y. and Rocque, P.A. (1990) J. Biol. Chem. **265**, 15361-15364
14. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllyla, R., Huntala, M., Koivu, J. and Kivirikko, K.I. (1987) EMBO J., **6**, 643-649
15. Wetterau, J.R., Combs, K.A., Spinner, S.N. and Joiner, B.J. (1990) J. Biol. Chem., **265**, 9800-9807
16. Cheng, S., Gong, Q., Parkison, C., Roberson, E.A., Appella,E., Merlino, G.T. and Pastan, I. (1987) J. Biol. Chem. **262**, 11221-11227
17. Morjana, N.A. and Gilbert, H.F. (1991) Biochemistry **30**, 4985-4990
18. Noiva, R., Freedman, R.B. and Lennarz, W.J. (1993) J. Biol. Chem. **268** 19210-19217
19. Wang, C-C. and Tsou, C-L. (1993) FASEB J. **7**, 1515-1517
20. Nigam, S.K., Goldberg, A.L., Ho, S., Rhode, M.F., Bush, K.T.and Sherman, M.Y. (1994) J. Biol.. Chem. **269**, 1744-1749
21. Puig, A. and Gilbert, H.F. (1994) J. Biol. Chem. **269**, 7764-7771
22. Macer, D.J.R. and Koch, G.L.E. (1988) J. Cell Sci. **91**, 61-70
23. Somlyo, A.P., Bond, M. and Somlyo, A.V. (1984) Nature, Vol. **314**, 622-625
24. Andrews, S.B., Leapman, R.D., Laudis, D.M.D. and Reese, T.S. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1682-1685
25. Lodish, H.F., Kong, N. and Wikstrom, L. (1992) J. Biol. Chem., **267**: 12753-12760
26. Lodish, H.F. and Kong, N. (1990) J. Biol. Chem., **265**, 10893-10899
27. Booth, C. and Koch, G.L.E. (1989) Cell, **59**,729-737
28. Sambrook, J. (1990) Cell, **61**: 197-199
29. Suzuki, C.K., Bonifacino, J.S., Lin, A.Y., Davis, M.M. and Klausner, R.D. (1991) J. Cell Biol., **114**, 189-205
30. Koch, G.L.E., Smith, M., Macer, D., Webster, P. and Mortara, R. (1986) J. Cell Sci.**86**, 217-232
31. Nguyen Van, P., Peter, F. and Söling, H-D. (1989) J. Biol. Chem. **264**, 17494- 17501