Biophysical Characterization of ERp29

EVIDENCE FOR A KEY STRUCTURAL ROLE OF CYSTEINE 125*

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ERp29 is a major resident of the endoplasmic reticulum (ER) that seemingly plays an important role in most animal cells. Although a protein-folding association is widely supported, ERp29’s specific molecular function remains unknown. A chaperone activity was postulated from evidence that ERp29 forms multimers like the classical ER chaperones, but conflicting results have emerged from our recent studies. Here a biophysical approach was used to clarify this issue and also reveal a key structural role for ERp29’s characteristic cysteine, Cys-125. Applying hydrodynamic parameters derived from sedimentation and dynamic light-scattering analyses, a model of ERp29’s quaternary structure was assembled from existing tertiary substructures. Comparison with Windbeutel, an ERp29-like protein from fruit fly with specialized chaperone activity, revealed similar tri-lobar gross structures but some finer differences consistent with functional divergence. Solubility and hydrophobic probe assays revealed moderate surface hydrophobicity, which was reduced in mutant ERp29 in which serine replaced Cys-125. This mutant was also relatively labile to proteolytic degradation, providing two reasons for the strict conservation of Cys-125. No multimerization was observed with untagged ERp29, which existed as tight homodimers (Kd < 50 nM), whereas His-tagged ERp29 artifically formed 670-kDa oligomers. These findings distinguish ERp29 biophysically from its peers in the ER including Windbeutel, endorsing our postulate that ERp29 adds a distinct type of folding activity to the ER machinery. By invoking novel functional associations for Cys-125 and the adjoining linker, new clues about how ERp29 might work have also arisen.

Proteins residing in the endoplasmic reticulum (ER) lumen, termed reticuloplasmins, hold broad significance in biology, medicine, and biotechnology. As central components of the ER machinery, reticuloplasmins play pivotal roles in the high quality production of secretory proteins, safe storage of calcium, and an associated range of serious health disorders (1–3). The most abundant reticuloplasmins include four ubiquitous “housekeepers” that help newly synthesized proteins fold by editing disulfide bonds (protein disulfide isomerase (PDI)) and acting as molecular chaperones (BiP, calreticulin, endoplasmic, PDI) (2, 4–6). Although these classical ER protein-folding assistants have well characterized individual roles, less is known about how they cooperate with one another and with additional ER constituents to ensure that diverse secretory proteins are folded correctly in different cell types. Compounding this issue is the recent discovery of several novel reticuloplasmins with uncertain functions (1, 7–9).

First characterized in 1997, ERp29 is now recognized as another major reticuloplasmin that might add distinct functionality to the ER machinery (10, 11). ERp29 is expressed ubiquitously in animal cells and with similar abundance as the four main folding assistants, implying a general housekeeping role (12–15). Concordantly, ERp29 has been encountered in a variety of physiological and pathological contexts, including normal production of dental enamel, milk, and antibodies, and disorders of the thyroid, spinal cord, and aging eye (11, 16–19). Although a protein-folding activity seems likely, ERp29’s specific molecular function remains unknown. Our studies have localized ERp29 primarily to the beginning of the secretory pathway (rough ER) and, by identifying various cell types that utilize ERp29 highly, implicated it in the production of hydrophobic proteins destined for membranes and export. ERp29 was also distinguished from its peers by regulatory and biochemical differences, including a distinctive lack of calcium binding activity (13, 15, 20, 21). Others have provided further evidence of such a distinct role, including ERp29’s novel tertiary structure (22), lack of classical ER stress-response elements (23), and ability to bind other ER proteins (17, 24–26). We recently purified ERp29 under native conditions and unexpectedly found no evidence of classical chaperone or PDI-like activities. This led to a revised hypothesis that ERp29 is a distinct type of folding assistant and highlighted some significant uncertainties about ERp29’s biophysical features (21).

Improved biophysical understanding of ERp29 is desirable for two prime reasons. Several gaps exist in current knowledge of ERp29’s structure, and these might be bridged by learning more about the associated hydrodynamic properties. Like other major reticuloplasmins, ERp29 has eluded attempts to determine its structure crystallographically (22, 27). Nonetheless, a major advance came with production of NMR-based structures for the two constituent domains (named N and C (10)) and evidence that the N domains are solely responsible for ERp29’s self-association as a dimer. Modeled assemblies of the N-C...
monomer and the dimerized N domains gave exciting insights to ERp29's novel structure, albeit significant uncertainties accompanied both inferred substructures (22). Indeed, the elongate homodimer implicit from combination of these part models conflicts with ERp29's reported behavior as a globular protein during size-exclusion chromatography (24, 25). Further questions have been raised by the crystallographic structure obtained recently for Windbeutel, an ERp29-like protein from fruit fly, which shows a quite different position for the dimer interface (27). Second, key functional attributes of ERp29 are likely to be reflected in its surface-based properties, yet studies to date have produced discordant results. Repeated evidence that ERp29 forms not only dimers but also higher order oligomers (multimers) was used aptly to promote a chaperone role (22, 28). However, such multimers were not observed in our studies of native ERp29 (15, 21). It is also unclear what significance should be attached to the generally weak interactions observed between ERp29 and other proteins in the ER (15, 17, 24–26, 29). Important functional insights have arisen from biophysical characterization of other reticuloplasmins (30–35), providing further incentive for such an investigation of ERp29.

This study sought to establish a hydrodynamically valid structural model for ERp29 and use it to evaluate relatedness with Windbeutel at the quaternary level. We also aimed to address the hypotheses that ERp29 is either a chaperone (22, 24, 26) or a distinct type of folding assistant (21) through characterization of its surface properties. Thirdly, the biophysical influence of ERp29's strictly conserved cysteine (Cys-125) was questioned because, with no evidence of involvement in a PDI-like activity (21), we postulated that Cys-125 might instead play an important structural role. With the availability of pure, tissue-derived (native) ERp29 limiting, our approach was to make a true, tag-free copy of the rat protein by recombinant expression and authenticate this using biochemical criteria established for native protein (12, 21). Our findings indicate that Cys-125 does indeed have structural importance and that ERp29 is biophysically distinct from its peers including Windbeutel, collectively supporting the notion of a distinct protein-folding activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sources of native ERp29, anti-ERp29, liver microsomes, and protein standards were as described (21). NDSB-201 (3-(1-pyridinio)-1-propane-sulfonate) was from Sigma, and ANS (8-anilino-1-naphthalenesulfonic acid) was from Fluka, and ANS (8-anilino-1-naphthalenesulfonic acid) was from Sigma. Restriction endonucleases were supplied by Roche Applied Science, Amersham Biosciences, and New England Biolabs.

**Production of Recombinant ERp29**

Wild Type rERp29—The pET 22b+ system (Novagen) was used to express mature rERp29 in the periplasm of Escherichia coli BL21(DE3). An ERp29 insert without signal sequence was PCR-amplified from the original rat ERp29 clone (pETErp29) and ligated into the MscI/HindIII-restricted vector, giving pProEx-ERp29 and pProEx-C125S, respectively. Following induction (0.6 mM isopropyl-1-thio-D-galactopyranoside, 3 h), conventional procedures were used to prepare cell lysates and isolate tagged proteins by nickel affinity chromatography on a chelating Sepharose column (Amersham Biosciences).

**Size-exclusion Chromatography**

Mini-scale chromatography on Sephacryl-S200HR (8-ml bed, 10 µg of rERp29 loaded in 0.1 ml) with internal calibration was as described (21). For higher loads (170 µg of rERp29 in 0.8 ml), samples were chromatographed (0.2 ml/min, 20 °C) on a 24-ml Superdex-75 column (Amersham Biosciences) equilibrated in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% isopropanol alcohol. External calibration and calculation of the Stokes radius, Rs, was done using the following standards: bovine serum albumin, 66 kDa; Rs = 3.7 nm; ovalbumin, 44 kDa, Rs = 3.05 nm; carbonic anhydrase, 29 kDa, Rs = 2.4 nm; cytochrome c, 12.4 kDa, Rs = 1.9 nm (36).

**Sedimentation Analysis**

For equilibrium analysis, samples (145 µl, 0.32 mg/ml in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol) were centrifuged (20,000 × g, 20 °C, An-60 Ti rotor) in a Beckman XL-A analytical Ultracentrifuge until equilibrium was achieved (∼24 h). Data were analyzed with SEDENG software, initially assuming a single sedimenting species (37). Calculations of solvent density and ERp29's partial specific volume (ν = 0.7433 cm³/g from composition) were made with SEDENTRP software, and ERp29 was quantified from A280 values using the molar extinction coefficient (25,480 M⁻¹ cm⁻¹ for monomer) calculated with the ProtParam tool (expasy.org; www.expasy.org). For sedimentation velocity analysis, samples (145 µl, 0.17 mg/ml in 10 mM phosphate buffer, pH 7.2) were centrifuged (40,000 × g, 20 °C) in the same instrument, and radial scans (230 nm, 4-min intervals) were analyzed with SEDFIT software (38).

**Dynamic Light-scattering Analysis**

Samples (12 µl, 1 mg/ml in 50 mM Tris-HCl, pH 7.2) were analyzed at 20 °C using a DynaPro 99E instrument and Dynamics software (Protein Software) after centrifugation to remove any gross particulates (15,000 × g, 10 min). Translational diffusion constants (D) were obtained directly from the rate of optical decay, and standard approaches were used to calculate Rs (Stokes-Einstein equation), frictional coefficient (Einstein-Sutherland equation), and M (assuming spherical protein with typical hydration, h = 1.3). Polydispersity was defined as one S.D. of Rs (Dynamics software).

**Hydrodynamically Valid Modeling of ERp29’s Quaternary Structure**

For comparison with “anhydrous” molecular models of ERp29, it was necessary to downscale the hydrodynamic dimensions of rERp29 (see Table I) since these represented hydrated protein (39, 40). The frictional ratio of a typical globular protein (f/f₀ = 1.2) is 0.2 higher than expected for an equivalent sphere because of bound water (41). Consequently, ERp29 was “dehydrated” by adjusting the principal dimensions of the ellipsoid to reduce f/f₀ by 0.2 (Perrin equation (41)). Structural modeling was done with MOLMOL software (42), using the NMR-based structures of ERp29's N and C domains (Protein Data Bank accession numbers 1g7d and 1g7e (22)) and the crystal structure of Windbeutel (Protein Data Bank accession 1g7d and 1g7e (22)) and the crystal structure of Windbeutel (Protein Data Bank accession number 1g7d (22)). The ERp29 quaternary model was refined to the dehydrated ellipsoid (f/f₀ = 1.17) by introducing additional interdomain “bend” toward the long axis and a minimal (0.4 nm) longitudinal “stretch” as needed to avoid conflict between the N and C domains. This refinement was permissible within the size of the periplasmic content (55%). α-helix, 11% β-strand, 34% other, calculated using the K2d tool at www.embl.de/−andrake/3d.html as expected (22).

**C125S Mutant**—The region surrounding Cys-125 was excised from pETErp29 (NdeI, Stul) and replaced with an equivalent PCR-amplified insert that coded for serine in place of Cys-125. The sequence-verified construct (pETERpCS) was used to produce C125S mutant protein as outlined for wild type rERp29, except that protease inhibitors (benzamidine, phenylmethylsulfonyl fluoride) were added at 4-fold higher concentrations and elution from the heparin-affinity column was modified as described below (see Fig. 6).
respectively. Surface electrostatic properties of ERp29 were analyzed using MOLCAD software (Trilos Inc.).

Solubility and Surface-hydrophobicity Assays

For the solubility assay, concentrated stock solutions of ERp29 (7–10 mg/ml) were precipitated by buffer exchange into 25 mM Tris-HCl, pH 7.2, 500 mM NDSB-201, using a centrifugal ultrafiltration device (Centricon C-10, Millipore). Dilution series were prepared in the same buffer, and then triplicated 20-μl samples of each starting concentration were dialyzed overnight against 10 mM Tris-HCl, pH 7.2 (4 °C) using Slide-A-Lyzer microdialysis units (Pierce). After centrifuging (15,000 × g, 15 min), soluble protein concentrations were determined using the Bradford binding microassay (Bio-Rad) and compared with controls (i.e. stock solution, before and after overnight storage at 4 °C). To assay surface hydrophobicity (32, 43), ERp29 and control proteins (5, 12, 15, 21) were incubated with 100 μM ANS for 2 h at 4 °C and then analyzed in a PolarStar spectrofluorimeter (BMG LabTech- nologies) using appropriate settings (350 nm excitation, 490 nm emission) and then analyzed in a PolarStar spectrofluorimeter (BMG LabTech- nologies) using appropriate settings (350 nm excitation, 490 nm emission). The fixed-wavelength capability of the fluorimeter precluded a quantitative analysis of the surface hydrophobicity (32, 43), ERp29 and control proteins (5, 12, 15, 21).

Other Procedures

Conventional procedures for SDS-PAGE, denaturing two-dimensional gel electrophoresis, immunoblotting, Edman microsequencing, and matrix-assisted laser desorption/ionization-time of flight mass spectrometry were as described (12, 15, 44). For cross-linking, ERp29 (0.2 mg/ml in 20 mM phosphate, pH 7.2, 150 mM KCl) was incubated with 0.6 mM BS3 (bis(sulfosuccinimidyl) suberate) for 30 min before SDS-PAGE, essentially as before (21). ERp29 was routinely quantified by SDS-PAGE with carboxy anhydride (calibrated by amino acid composition analysis) as standard (21). Grand average hydropathicity (GRAVY (45)) of the interdomain linkers (see Fig. 7D) (TPLLYGDRGCL for Windbeutel (27)) was calculated using the ProtParam tool. Statistical comparisons of paired mean values were done with Student’s t test (two-tailed, homoscedastic).

RESULTS

Preparation of Authentic ERp29 by Recombinant Expression in E. coli Periplasm—To replicate ERp29 faithfully, we used a periplasmic expression approach whereby the correct N terminus was generated naturally by cleavage of signal peptide from exported ERp29 precursor. Such a prokaryotic expression system was deemed suitable because no post-translational additions were detected in native ERp29 (12, 21). After isolation using standard anion-exchange and heparin-affinity steps (21), rERp29 had high purity as assessed by SDS-PAGE (Fig. 1A), dynamic light scattering (see Fig. 3), Edman analysis, and mass spectrometry. When compared with native ERp29 (cf. (21)), rERp29 exhibited the same characteristic features of heparin binding and cross-linking as a 51-kDa dimer (not shown), low molecular mass by size-exclusion chromatography (40,000 versus 51,000, M expected) and high molecular mass by SDS-PAGE (29 kDa versus theoretical 25.6 kDa) (Fig. 1A, Table 1). Moreover, on denaturing two-dimensional gels, rERp29 and native ERp29 had indistinguishable major species (pI 6.0), indicative of sequence identity. Acidic isoforms were also present in rERp29 (Fig. 1B), supporting our earlier interpretation that ERp29 is prone to partial deamidation (12). Consequently, rERp29 was considered an authentic substitute for native ERp29 in the biophysical analyses that follow.

Hydrodynamic Properties of rERp29 Indicate a Nonglobular and Tightly Associated Homodimeric Structure—Previous studies, using size-exclusion chromatography and cross-linking, consistently identified ERp29 as a homodimer but conflicted over ERp29’s globularity and propensity to form multimers (15, 21, 24, 25). Using internally calibrated size-exclusion chromatography on Sephacryl-S200HR, rERp29 behaved as a monodisperse 40,000 M, species with a Stokes radius of 2.9 nm (Table 1). The same result was obtained on Superdex-75 and at 5-fold higher in-column concentrations of rERp29, verifying that this idiosyncratic behavior was not an artifact of matrix interactions or partial dissociation (not shown). Consequently, rERp29’s shape appeared more compact than that of a 51-kDa globular protein, as reported for native ERp29 (15, 21).

Sedimentation equilibrium analysis was used to obtain a native mass measurement unaffected by protein shape (46). At equilibrium, rERp29 behaved as a monodisperse 52-kDa species, closely matching the expected homodimeric mass (Fig. 2, upper panel). Data analysis was performed to evaluate the homodimerization affinity since this had not been quantified in native conditions before. When modeled as 100% dimer (i.e. totally associated), data residuals were distributed symmetrically about the baseline even at the lowest protein concentrations (~670 nm, ~6.9-cm radial distance), whereas an obvious skewing occurred with the 10% dissociation model (Fig. 2, lower panel). Taking 5% dissociation as the detectable limit, the total association of 670 nM dimer indicated strong binding, with K exceeding 50 nm.

Dynamic light-scattering analysis was applied as a complementarity approach that advantageously maintains a constant concentration of protein throughout (46). Again, rERp29 exhibited a monomodal size distribution (Fig. 3), albeit the moderately broad peak width (~27% polydispersity) pointed to some conformational or other heterogeneity. A primary measure of the diffusion constant was obtained from this analysis (Table 1), which also attested the high purity of the rERp29 (~99% by mass, two preparations tested).
detection limit for dissociation of /H11021 which matched expectations for a typical 51-kDa protein (13532). Characteristically low Stokes radius in size-exclusion chromatography was reconciled by the light-scattering value, which matched expectations for a typical 51-kDa protein (3.4 nm, assuming globularity). Moreover, the sedimentation constants derived from light-scattering and sedimentation equilibrium data were identical to that obtained directly by sedimentation velocity analysis (3.4 s; not shown). Based on the derived frictional ratio (\(f/f_o = 1.4\)), rERp29’s hydrodynamic behavior clearly differed from that of a globular protein.

No evidence of multimerization was observed during size-exclusion, cross-linking, sedimentation, or light-scattering analyses. The absence of multimers during dynamic light-scattering analysis is particularly significant because this technique is highly sensitive to larger masses. Modeling of the sedimentation equilibrium data (cf. Fig. 2) also indicated that low order multimers would have been detected with reasonable sensitivity (\(\geq 2.5\%\) of total, for trimers and tetramers). However, the sedimentation results did imply a solubility limitation (\(\geq 0.6 \text{ mg/ml}, 7.15 \text{ radial cm}\)) since at equilibrium, only about 20% of the loaded sample remained soluble, consistent with an earlier report that native ERp29 undergoes precipitation when concentrated above \(-1 \text{ mg/ml}\) (21).

Together these findings indicated that rERp29 behaved exclusively as a tightly associated homodimer that was both moderately elongate and compact. ERp29’s tendency to precipitate in vitro was investigated further because of its relevance to interactions with other proteins in vivo.

ERp29 Has Limited Solubility Despite Overall Hydrophilic Character—ERp29 is regarded as a soluble protein (reticuloplasmin) because it partitioned to the cytosolic fraction of tissue homogenates, and its predominantly hydrophilic sequence includes a canonical ER retention motif (10, 15). However, at higher concentrations, purified ERp29 underwent substantial losses during centrifugal ultrafiltration unless a nonionic detergent was present (21). This property may reside in the N domain, which was reported to have a hydrophobic surface patch and to be poorly soluble when separated from the C domain (22).

To quantify ERp29’s solubility, we developed an equilibrium assay based on a classical salting-in approach (47). In essence, rERp29 was concentrated to various levels in the presence of a mild solubilizing agent and, after removal of solubilizer by dialysis, the amount of rERp29 remaining soluble was measured. A nondetergent sulfobetaine (NDSB-201) was used as solubilizer because, not forming micelles like nonionic detergents, it is readily dialyzable. In the presence of 500 mM NDSB-201, rERp29 was completely soluble at 10 mg/ml (not shown), whereas after dialysis, rERp29 was only partially recovered in the soluble fraction (Fig. 4A). The losses showed a quasilinear relationship with protein concentration and were significantly greater than those observed with a highly soluble control, serum albumin (Fig. 4A). A visible film formed on the dialysis membrane at higher concentrations, consistent with interfacial precipitation of rERp29.

We next measured binding of the environment-sensitive fluorophore, ANS, to evaluate ERp29’s solubility limitation in context of surface hydrophobicity. ANS has been widely used as a hydrophobic probe, including with ER chaperones (32, 48). When compared with a range of soluble proteins (Fig. 5), rERp29 exhibited moderately high hydrophobic character, being similar to ovalbumin, which contains three ANS-binding sites (43). Although not challenging the classification as a soluble protein, these results implied that ERp29 has multiple hydrophobic features, at least one of which can limit its solubility in vitro.

Cysteine 125 Has an Important Structural Role but Does Not Influence Solubility—Having eliminated Cys-125 from a PDI-like catalytic role (21), we asked whether this characteristic residue might instead hold structural significance for ERp29. Notably, cysteine can impart strong hydrophobicity in situ (49), and spin-labeled Cys-125 was implicated as a major binding contact in ERp29 multimers (22).
To address this hypothesis, a site-directed mutant was made with serine replacing Cys-125 (C125S mutant). The C125S mutant was essentially indistinguishable from wild type rERp29 as measured by SDS-PAGE (Fig. 1A), solubility (Fig. 4B), and hydrodynamic analyses (size-exclusion chromatography, sedimentation equilibrium, and dynamic light scattering; cf. Table I). Similarly limited solubilities were apparent for rERp29 and the C125S mutant, whereas His-rERp29's solubility approached that of serum albumin.

Intriguingly, the C125S mutant was consistently found to be partially degraded during purification (Fig. 6), unlike wild type rERp29 and native ERp29 (21). Edman analysis of the major and minor fragments (16-kDa doublet) revealed intact N-terminal sequences (LHTK...), and the C-terminal-cut sites were localized to alanine 139 and alanine 133 by mass spectrometry (Fig. 7D and data not shown). Immunoblot analysis showed that this C-domain truncation (C125S') occurred only after the periplasmic extract was prepared. Moreover, C125S' remained stable after purification, suggesting that it had been effectively separated from a protease such as elastase (which cuts preferentially after alanine). It was also found that a C125S'-containing species could be separated from intact homodimer by heparin-affinity chromatography (Fig. 6 and data not shown), implying that the C domain is largely responsible for ERp29's heparin binding property. Together these effects of the C125S mutation on proteolytic stability and ANS binding confirmed our hypothesis, but a major role for Cys-125 in self-association (22) or solubility of ERp29 was not supported.

His-tagged ERp29 Is Prone to Artifactual Oligomerization—It has been reported that a substantial proportion of His-tagged ERp29 (>20% based on size-exclusion chromatography) exists as soluble multimers, pointing to a probable chaperone-like activity (22, 28). To investigate whether this important discrepancy reflected our use of untagged ERp29, we prepared a His-tagged variant of rERp29 (His-rERp29). The N-terminal addition of a widely used tag (comprising 25 mainly hydrophilic residues; ProEx system) gave a small increase in $M_r$ during SDS-PAGE as expected (Fig. 1A). Surprisingly however, His-rERp29 behaved much larger than its untagged coun-

FIG. 4. rERp29 has limited solubility in vitro. A, solubility of rERp29 was assessed before and after removal of NDSB-201 solubilizer (filled circles, start and finish concentration, respectively). At higher concentrations, ~30% of rERp29 was absent at the finish. In contrast, recoveries of bovine serum albumin (BSA, open circles) approached ideality (broken line) more closely. B, comparative analysis of solubilities at 5 mg/ml starting concentration, expressed as a percentage of serum albumin (±S.D.). Similarly limited solubilities were apparent for rERp29 and the C125S mutant, whereas His-rERp29's solubility approached that of serum albumin.

FIG. 5. ANS binding indicates that the C125S mutant has less surface hydrophobicity than wild type rERp29. ANS fluorescence (arbitrary units; ±S.D.) for rERp29 and the C125S mutant, compared with control proteins ranging from highly hydrophilic (amylase, RNase, carbonic anhydrase (Carb Anhyd)) through moderate surface hydrophobicity (ovalbumin), are shown.

FIG. 6. The C125S-mutant is susceptible to proteolytic fragmentation of the C subunit. A, absorbance trace ($A_{280}$) from heparin-affinity chromatography, in which a modified step gradient (broken line) was used to separate partially degraded from intact C125S-mutant ERp29 (early and late peaks, respectively). B, Coomassie-stained SDS-PAGE of the indicated fractions from A, showing that the 16-kDa fragment (C125S') appeared in the early peak only. Edman analysis of the C125S' band (appearing variably as a doublet) showed the N terminus was intact (see "Results"). Since a similar separation from intact C125S protein was achieved with size-exclusion chromatography, the early peak apparently comprised C125S homodimers with one truncated C domain. No such fragmentation was observed with rERp29.
Fig. 7. A hydrodynamically valid model of ERp29’s quaternary structure. A, the refined model of ERp29, as assembled from NMR-based models of the N and C domains and the N–N dimer (22) and adjusted to better fit the ellipsoid defined here from rERp29’s hydrodynamic parameters (Tables I and II). The two views are separated by 90° rotation about the long axis. As depicted schematically (blue bar), the modeled ERp29 homodimer has a tri-lobe structure comprising a central N lobe (≈ two N domains) with a C lobe (≈ C domain) at each end. Cys-125 and Ala-139 are colored yellow and red, respectively. Proximity of the N terminus (arrow) to the dimerization interface is noteworthy, particularly since the first 14 residues (i.e., the His tag and N-terminal dipeptide) were missing from the data set (22). B, an equivalent portrayal of Windbeutel, based on the crystallographic structure (27). Windbeutel appears grossly equivalent to but more globular than ERp29, and its dimerization interface is reversed with respect to that of ERp29. C, alternative models of ERp29 (Δ ERp29), showing that a back-to-back dimerization of ERp29, akin to Windbeutel, is compatible with the hydrodynamic shape, whereas a parallel alignment of monomers is not. D, schematic illustrating that the primary proteolytic cut site defined here for C125S (alanine 139, red circle) is located in an unstructured loop separating two α-helical segments (H5, H6) of the C domain (cf. scissors in A). The hydrophobic character of the interdomain linker is also depicted (green and yellow circles, hydrophobic residues).

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Derivation of a Structural Model for Native ERp29—With seemingly robust hydrodynamic parameters at hand, we next sought to apply this information to the construction of a quaternary structural model for ERp29. Mkrtchian and co-workers’ seminal NMR study (22) provided the requisite building blocks, that is, experimentally determined structures for the N and C domains and a data-guided assembly of the N-domain dimer. Starting with their provisional monomer construct, we assembled the homodimer based on the reported N-domain interface and antiparallel orientation of monomers. Next we adjusted the hydrodynamically derived measurements of rERp29’s ellipsoid (Table I) to enable comparison with the anhydrous molecular structure. To do this “dehydration,” a typical hydration level was assumed for ERp29 (Table II). Third, we analyzed how the main uncertainties of the provisional model (co-linearity of N and C domains in monomer, rotation at the dimer interface) affected its overall shape. A generally good dimensional overlap was obtained between the initial NMR-derived assemblage and the ellipsoid calculated for dehydrated ERp29, whereas small variations in rotation or interdigitation at the dimer interface resulted in noticeably poorer fits. Moreover, an alternative model with parallel monomers was ruled out by its much more globular shape (Fig. 7C). After minor realignment of the C domains, the ERp29 model showed close correspondence to the hydrodynamically derived ellipse, albeit being somewhat less elongate (Fig. 7A, Table II). Consequently, we adopted this refined assembly as a hydrodynamically valid working model for native ERp29.

ERp29 and Windbeutel Have Distinct Shapes—Recently, a complete structure of the fruit fly protein, Windbeutel, was derived by crystallography, but uncertainties surrounding the NMR-based model of ERp29 thwarted comparisons at the quaternary level (27). The exciting elucidation of Windbeutel’s role as an ER/Golgi escort-chaperone (27, 50, 51) therefore remains of uncertain relevance to ERp29. Comparison with the hydrodynamically refined model of ERp29 (Fig. 7, A and B) revealed that, although equivalent in overall domain arrangement, Windbeutel was noticeably more globular (12% shorter, 16% wider). A second major difference was the opposite orientation of N domains at the dimer interface, as noted (27). When ERp29 was remodeled in a back-to-back orientation like Windbeutel, it again fitted well within the hydrodynamic ellipse (Fig. 7C), making this a plausible alternative to the NMR-
DISCUSSION

A pressing need exists for better functional understanding of ERp29, a major resident of the ER with seemingly general importance in animal cells. In turn, a deeper knowledge of ERp29’s structural properties is required, being a prime source of functional clues. Toward these goals, we report the first comprehensive biophysical analysis of ERp29, using authenticated recombinant protein and a site-directed mutant that lacks the sole cysteine, Cys-125. The results indicate for the first time that Cys-125 plays a key structural role by contributing to the stability of the C domain and hydrophobicity of the interdomain linker. Both of these properties have potential functional significance and so might account for the evolutionary conservation of Cys-125. Second, ERp29 was found exclusively as an elongate homodimer whose exposed hydrophobic features and conformational flexibility endorse a protein-folding role. Previous reports that ERp29 undergoes chaperone-like multimerization (22, 28) were not supported. Third, using the derived hydrodynamic parameters to refine a quaternary structural model, ERp29 was found to have notable structural differences from its distant relative, Windbeutel. This result helps to explain emerging evidence that these proteins have diverged functionally despite their overall structural similarity. Our findings collectively support the proposition that ERp29 is a distinct type of folding assistant and, by focusing attention on Cys-125 and the C domain, provide valuable new insights that these proteins dimerize in opposite orientations as indicated by the current models and to detail the relative positioning of ERp29’s lobes when the linker is intact. Such studies should be done with proteins lacking N-terminal tags given their likely proximity to the dimerization site and the evidence here that His-rERp29 did not dimerize stably like rERp29. It also remains to be seen whether the conformational differences have behavioral ramifications, such as underpinning ERp29’s inability to process nascent Pipe protein and Windbeutel’s tractability for crystallographic analysis (27, 51). Interestingly, although shape differences are apparent for both the N and the C lobes, ERp29’s C domain complemented its Windbeutel counterpart in domain-swapping experiments, whereas the N domain did not (27). These comparisons (Fig. 7) are limited by uncertainties regarding ERp29’s actual hydration level (recognizing only a modest influence on outcome, we assumed a typical value for soluble proteins (39–41)), the effect of pH on ERp29’s structure (NMR data were collected at pH 4.9 (22)), and whether Windbeutel’s conformation in physiological solution is accurately portrayed by the crystal structure (e.g. calmodulin is a prime case in which this does not hold (54)). Hopefully, these limitations will be addressed in future studies through provision of hydrodynamic data for Windbeutel and a crystal structure for ERp29. It is noteworthy that, through dimerization, ERp29 achieves a hydrodynamic footprint similar to that of Erp60, a 55-kDa homolog of PDI (34).

We found that rERp29 had limited solubility in vitro, consistent with moderate surface “stickiness,” and that the dimerization affinity was at least 20-fold higher than reported previously (22). The latter estimate (Kd = 1 μM) was made by electrospray ionization mass spectrometry, a procedure less likely to preserve physiological associations than the sedimentation approach used here (Fig. 2). With a Kd < 50 nM and luminal concentrations 3 orders higher (~100 μM, similar to PDI and endoplasm (15, 32, 55)), ERp29’s dimer can be expected to remain fully associated in vivo. The limited solubility of rERp29, quantified here for the first time, is consistent with moderate hydrophobicity apparent from ANS binding (Figs. 4 and 5) and the scattered areas of hydrophobic exposure seen with surface modeling (22). Ionic interactions were also implicated because higher salt concentrations increased rERp29’s solubility, like NDDB-201 and nonionic detergent2 cf. Fig. 4). Without these solubilizing agents, purified ERp29 seemingly has a solubility threshold (~1 mg/ml, 20 μM dimer) above which it precipitates, as seen here during sedimentation and solubility analyses. This self-aggregation tendency is unlikely to manifest physiologically, however, because although the luminal concentration of ERp29 might exceed 20 μM, ERp29’s stickiness should be quenched by the protein-solubilizing properties of neighboring reticuloplasmins (56). Further investigation is justified to establish whether ERp29 interacts preferentially with any of its cellular neighbors, consistent

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with a functional partnership. However, evidence that precipitation was prevented by a very mild solubilizing agent (NDSB-201 (57)) suggests that any such interactions will be transient, as can be inferred from several other results (15, 26, 29). Interestingly, a predisposition to amyloid formation is predicted for ERp29 by the Tango tool (tango.embl.de (58)), raising the possibility that its solubility limitation could be manifest in pathological contexts.

Our evidence, that the C125S mutation reduced rERp29's surface hydrophobicity and proteolytic stability, implicates Cys-125 in a defining role that has been preserved through evolution. Using limited trypsinolysis, we have verified that the C domain is less stable in the C125S mutant than its wild type counterpart7. Both with the initial elastase-like cleavage at alanine 139 (Fig. 6) and with trypsin, the destabilizing effect appears due to allosteric exposure of a loop that is separated from Cys-125 by a short helical segment (Fig. 7D). Since cleavage at this loop effectively ablates the C domain and no such fragmentation was seen in fresh tissue samples (12, 15), it follows that maintenance of ERp29's structural stability could be one selective pressure on Cys-125. A second reason for conservation emerged from the finding that Cys-125 affected ANS binding (Fig. 5). The simplest interpretation, that Cys-125 constitutes an ANS-binding site, is consistent with the knowledge that cysteine can add substantial hydrophobicity to folded proteins (49) and the evidence here that rERp29's gross hydrodynamic properties were unaffected by the C125S mutation. This result draws attention to several other hydrophobic residues adjoining Cys-125 (Fig. 7D), suggesting the interdomain linker is not only flexible (22) but also "sticky." As our results did not support involvement in multimerization (22), we propose that the linker might instead be involved in substrate interactions being strategically placed at the border of a cleft between the N and C lobes (Fig. 7). Supporting this idea, ERp29's linker is considerably more hydrophobic than that of Windbeutel (GRAVY scores of 1.2 and 0.2, respectively (45)), consistent with the apparent differences in substrate specificity (27) and the suggestion that ERp29's preferred substrates are hydrophobic (13, 20).

Collectively, the observed biophysical characteristics support our working hypothesis that ERp29 is a distinct type of folding assistant that differs mechanistically from classical chaperones. The consistent lack of soluble multimers observed throughout this study distinguishes ERp29 from major chaperones in the ER (31, 33-35, 49, 59) and elsewhere (46) and accords with the lack of activity in conventional chaperone assays (21). It is possible that conflicting reports of multimerization reflected the use of N-terminally tagged ERp29, particularly since multimers were not obvious after cross-linking of endogenous ERp29 in cultured cells (22, 24). However our findings cannot be compared directly because His-rERp29 contained a longer tag than that employed by the others (25 and 12 residues, respectively, both attached to rat ERp29). Tight dimerization is an additional characterized feature that sets ERp29 apart from the other major reticuloplasmins. In support of a folding role, ERp29's tri-lobe structure has overall similarity not only to Windbeutel but also other putative chaperones that contain mobile domains involved in substrate trapping, and the coiled-coil nature of ERp29's C domain resembles the substrate-binding elements used in some recently defined chaperones (60-62). Conformational flexibility, an enabling feature of folding assistants (30, 33), is also implicit from ERp29's proteolytic lability. The N lobe's resistance to degradation confirms that the C lobes are relatively loosely folded and mobile, as inferred from NMR analysis (22). It follows that the C lobes are appropriately configured to clamp nascent-protein substrates against the stable N lobe and hydrophobic linker, thereby facilitating folding in some way. Other hydrophobic sites, as implicit from the ANS binding and solubility results, provide the basis for further interactions with substrates and partners.

In conclusion, this biophysical characterization has filled some functionally important gaps in knowledge about the quaternary structure and surface-based properties of ERp29. We now have a stronger foundation to visualize how ERp29 looks in solution and so contemplate its putative interactions with nascent secretory and membrane proteins. It seems plausible that the elongate homodimer provides an extended platform for folding, perhaps assisted by trapping substrates in the two interdomain clefts. Moreover, the observed biophysical divergence from Windbeutel (globularity, hydrophobic linker, acidic cleft) highlights the uncertain evolutionary relationship of these proteins and further questions whether they are functional homologs as originally supposed (27, 63). Secondly, with improved understanding of how ERp29 behaves in solution, attention can now be shifted from multimerization (22, 28) to a more general need for stabilization that is provided naturally by other ER proteins. Thirdly, this study has provided stimulating clues about how ERp29 might work by revealing the potential for the linker to act as a substrate interaction site and for Cys-125 to regulate stability of the C lobes. We have recently found that Cys-125 is both highly reactive and accessible to a physiological redox regulator, raising the intriguing possibility that ERp29 is affected by redox conditions in the ER.3 Finally, although introducing caution over use of N-terminal tags, our results hint that ERp29's default conformation (Fig. 7) might be altered by regulatory or functional interactions. Not only was dimerization disrupted in His-rERp29, but macromolecular interaction with the hydrophobic linker could necessitate conformational rearrangement of adjoining C lobes (Fig. 7).3 Valuably, this study endorses our postulate that ERp29 adds a distinct folding activity to the ER machinery and helps shape plans for resolving the conundrum of ERp29's specific molecular role.

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REFERENCES

1. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Science 286, 1882-1888
2. Richalak, M., Robert Parker, J. M., and Opas, M. (2002) Cell Calcium 32, 269–278
3. Kim, P. S., and Arvan, P. (1998) Endocrinology 19, 173–202
4. Freedman, R. B., Klaппa, F., and Ruddock, L. W. (2002) EMBO Rep. 3, 136–140
5. Gething, M. J. (1999) Semin. Cell Biol. 10, 465–472
6. Argen, Y., and Simen, B. B. (1999) Semin. Cell Biol. 10, 495-505
7. Aiken, H. I., Williamson, B. A. Howard, M. J., Lappi, A. K., Jantti, H. P., Rautio, S. M., Kellonkumpu, S., and Ruddock, L. W. (2005) J. Biol. Chem. 278, 28912–28920
8. Anelli, T., Alessio, M., Bachi, A., Bergamelli, L., Bertoli, G., Camerini, S., Menghini, A., Ruffo, E., Simmen, T., and Sittia, R. (2005) EMBO J. 22, 5015–5022
9. Knoblauch, B., Keller, B. O., Groenendyk, J., Aldred, S., Zheng, J., Lemire, B. D., Li, L. and Richalak, M. (2003) Mol. Cell. Proteomics 2, 1104–1119
10. Demmer, J., Zhou, C., and Hubbard, M. J. (1997) FEBS Lett. 402, 145–150
11. Hubbard, M. J. (2002) Proteins 49, 1069–1078
12. Hubbard, M. J., and McCugh, N. J. (2002) Trends Genet. 18, 362–365
13. Hubbard, M. J., McCugh, N. J., and Carne, D. L. (2000) Eur. J. Biochem. 267, 1945–1957
14. van Anken, E., Romijn, E. P., Maggioni, C., Menghini, A., Sittia, R., Brackman, I., and Heck, A. J. (2005) Immunity 18, 243–253
15. Baryshev, M., Sargsyan, R., Vallin, G., Lejnieks, A., Furudate, S., Hishinuma, M. J. Hubbard and J. E. Mangum, unpublished data.

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