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Properties of Soluble Fusions Between Mammalian Aspartic Proteinases and Bacterial Maltose-Binding Protein

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The mammalian aspartic proteinases procathepsin D and pepsinogen form insoluble inclusion bodies when expressed in bacteria. They become soluble but nonnative when synthesized as fusions to the carboxy terminus of *E. coli* maltose-binding protein (MBP). Since these nonnative states of the two aspartic proteinases showed no tendency to form insoluble aggregates, their biophysical properties were analyzed. The MBP portions were properly folded as shown by binding to amylose, but the aspartic proteinase moieties failed to bind pepstatin and lacked enzymatic activity, indicating that they were not correctly folded. When treated with proteinase K, only the MBP portion of the fusions was resistant to proteolysis. The fusion between MBP and cathepsin D had increased hydrophobic surface exposure compared to the two unfused partners, as determined by bis-ANS binding. Ultracentrifugal sedimentation analysis of MBP–procathepsin D and MBP–pepsinogen revealed species with very large and heterogeneous sedimentation values. Refolding of the fusions from 8 M urea generated proteins no larger than dimers. Refolded MBP–pepsinogen was proteolytically active, while only a few percent of renatured MBP–procathepsin D was obtained. The results suggest that MBP–aspartic proteinase fusions can provide a source of soluble but nonnative folding states of the mammalian polypeptides in the absence of aggregation.

KEY WORDS: Aspartic proteinases; protein folding; protein fusions.

1. INTRODUCTION

Cathepsin D, pepsin, and renin are members of the aspartic proteinase family and share structural features. They are synthesized in the mammalian endoplasmic reticulum (ER)[3] with propeptides of about 45 amino acids. The signal peptides that are required for translocation across the ER membrane are removed cotranslationally, and as the polypeptide grows in length it is believed to interact with folding catalysts in the lumen of the ER (Nilsson and Anderson, 1991). Procathepsin D is proteolytically processed via several steps to the mature form on the way to and within the lysosome (Hasilik and Neufeld, 1980; Hasilik, 1992; Delbruck *et al.*, 1994). Procathepsin D is N-glycosylated, while glycosylation of pepsinogen and prorenin is variable and species specific.

When expressed in *Escherichia coli*, the aspartic proteinases accumulate in inclusion bodies. Pepsinogen is readily renatured after solubilizing inclusion bodies in urea and refolding *in vitro* (Lin *et al.*, 1989, 1995; Cottrell *et al.*, 1995). In contrast, procathepsin D forms mostly insoluble aggregates during refolding, with only a few percent recovery of native protein (Conner and Udey, 1990; Scarborough and Dunn, 1994). This has made it difficult to study recombinant procathepsin D.

The aspartic proteinases are bilobed molecules which show a conserved pattern of secondary structure, with the active-site cleft located between the two lobes.

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[3] Abbreviations: ER, endoplasmic reticulum; bis-ANS, 1,1'-bis(4-anilino)naphthalene-5,5'-disulfonic acid; CpD, cathepsin D; MBP, maltose-binding protein; Pgn, pepsinogen; PMSF, phenylmethane sulfonyl flouride.

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The successful prediction and modeling of the structures of members of the family based on known three-dimensional coordinates of other members (Koelsch *et al.*, 1995) suggest that a basic folding pathway is probably shared by the members of the family. The aspartic proteinases have a high proportion of β-sheet structure, which may favor inclusion body formation when they are expressed in *E. coli*.

We have previously investigated the bacterial expression of the aspartic proteinases as fusion proteins with *E. coli* partners such as maltose-binding protein (MBP) and thioredoxin. When the bacterial partners were fused to the C-terminus of aspartic proteinases, the fusion proteins accumulated in insoluble inclusion bodies (Sachdev and Chirgwin, 1998*a*). However, when MBP was moved to the N-terminus of the aspartic proteinase, the MBP-aspartic proteinase fusion proteins were expressed at high levels in the cytosol of *E. coli* as soluble and stable proteins (Sachdev and Chirgwin, 1998*b*). However, the soluble fusions lacked detectable proteinase activity, suggesting that the aspartic proteinase portions were nonnative. Therefore, we undertook physicochemical characterization of these fusions, both as isolated from the bacterial cytosol and after refolding *in vitro*.

2. EXPERIMENTAL PROCEDURES

2.1. General Methods

All reagents were of analytical grade and obtained from Sigma (St. Louis, MO) or New England Biolabs (Beverly, MA). All DNA constructs have been described previously (Sachdev and Chirgwin, 1998*b*). The enzymatic activities of the aspartic proteinases were determined by digestion of labeled hemoglobin into peptides soluble in 3% trichloroacetic acid (Sachdev and Chirgwin, 1998*a*). Human cathepsin D, a two-chain glycoprotein, was purified from human placentas by pepstatinyl agarose affinity chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 1.5-mm-thick, 12.5% or 10% acrylamide slab gels according to Laemmli (1970). Molecular weight standards were bought from Sigma (St. Louis, MO). Gels were stained with 0.1% Coomassie brilliant blue R250 in 40% methanol and 10% acetic acid. Gels were destained with 40% methanol and 10% acetic acid. Protein concentrations were determined by the Bradford method using Bio-Rad (Richmond, CA) protein assay dye reagent.

2.2. Pepstatinyl Agarose Binding

MBP–procathepsin D and MBP–pepsinogen were expressed in *E. coli* and purified to greater than 90% homogeneity as described (Sachdev and Chirgwin, 1998*b*). Refolded MBP–procathepsin D and MBP–pepsinogen, 50–75 μg, were adjusted to 0.1 M sodium acetate (pH 3.5), 0.1% Brij 35, and 1 M NaCl. Pepstatinyl agarose (Pierce, Rockford, IL) was washed three times with the same buffer. The protein was mixed with 50 μl of a 50% slurry of pepstatinyl agarose overnight at 4°C. The resin was pelleted by centrifugation in a microcentrifuge at full speed for 1 min. The supernatant was saved as the unbound fraction. The agarose was washed 3 × with 1-ml aliquots of buffer containing 0.1 M acetate (pH 3.5), 0.1% Brij 35, and 0.35 M NaCl. The bound fraction was eluted with 2 × 500-μl aliquots of 50 mM Tris (pH 8.6), 0.1% Brij 35, and 1 M NaCl. The unbound fraction, wash fraction, and bound fraction were acetone precipitated prior to SDS–PAGE analysis to reduce volume. They were analyzed by SDS–PAGE on 10% polyacrylamide gels followed by Coomassie blue staining. To control for nonspecific binding, assays were also performed in parallel in the presence of soluble pepstatin added to the samples prior to mixing with pepstatinyl agarose.

2.3. Protease Digestion

Ten μg each of MBP–procathepsin D and MBP–pepsinogen in 0.1 M phosphate buffer (pH 7.4) and 5 mM EDTA was digested with 0.01 μg (0.1% w/w) or 0.1 μg (1% w/w) proteinase K for 1 hr at 22°C in 0.1 M phosphate buffer (pH 7). Proteolysis was stopped by adding 50 μg of PMSF from a 1 mg/ml stock and boiling the sample. As a control to ensure that the proteinase K was completely inactivated, aliquots of each fusion were also analyzed by adding 10 μg of fusion protein to similarly inactivated proteinase K. Laemmli sample buffer (2 × concentrated) was added to all the samples, and they were analyzed by SDS–PAGE.

2.4. Bis-ANS Binding to MBP–Cathepsin D

Fluorescence of 1,1'-bis(4-anilino)naphthalene-5,5'-disulfonic acid (bis-ANS) was measured using an SLM Model 500C fluorometer at an excitation wavelength of 397 nm after adding 10 μM bis-ANS to 1 mg/ml protein. Solutions were mixed thoroughly after addition of bis-ANS and the emission spectra recorded over the range 400–600 nm. bis-ANS binding to placental cathepsin D

and to MBP was compared to its binding to the fusion protein, MBP–cathepsin D.

2.5. Sedimentation Velocity Analysis by Analytical Ultracentrifugation

Sedimentation velocity analysis of the various proteins was performed in a Beckman XL-A analytical ultracentrifuge. The protein concentrations were between 0.2 and 0.4 mg/ml. The temperature was kept constant at 25°C. The rotor speed varied from 8000 to 30,000 rpm depending on the sample. The scans were analyzed by the method of van Holde and Weischet (1978) using the UltraScan ultracentrifuge data collection and analysis program (B. Demeler, Department of Biochemistry, University of Texas Health Science Center). Data were corrected for buffer density and viscosity. The samples were dialyzed against either 0.1 M phosphate buffer (pH 7.0) and 5 mM EDTA, or 10 mM Tris (pH 7.4) and 100 mM NaCl. Refolded fusions were analyzed in the former buffer. All of the sedimentation velocity runs were performed at the Center for Analytical Ultracentrifugation of Macromolecular Assemblies, Department of Biochemistry. The analysis of the sedimentation velocity runs is shown as the integral distribution of $s_{20,w}$ for the protein. The y axis indicates the fraction of sample with $s_{20,w}$ values less than or equal to the values on the x axis ($s_{20,w}$ values).

2.6. Refolding of Purified MBP–Aspartic Proteinase Fusion Proteins

Fusion proteins were refolded as described by Kuhelj *et al.* (1995), who described the successful refolding from bacterial inclusion bodies of procathepsin B, a lysosomal cysteine proteinase. MBP–procathepsin D, MBP–cathepsin D, and MBP–pepsinogen were expressed as soluble proteins in *E. coli*, purified by amylose resin affinity chromatography, and equilibrated in 0.1 M phosphate (pH 7.0), 5 mM EDTA to a protein concentration of 500 µg/ml. They were denatured by addition of solid urea to 8 M and incubation for 4–6 hr at room temperature. The denatured proteins were refolded by dialysis for 15 hr against 500 volumes of refolding buffer, 0.1 M phosphate (pH 7.0), 5 mM EDTA, and 5 mM cysteine, at 4°C. The cysteine was removed by dialysis for 2 hr against 500 volumes of the same buffer without reducing agent. After dialysis, the protein was transferred to a polypropylene tube and centrifuged at 6000 × g for 10 min to remove particulates.

2.7. Solubility of MBP–Procathepsin D at various urea concentrations

MBP–procathepsin D and unfused MBP were expressed, purified by affinity chromatography on amylose resin, and equilibrated in 50 mM HEPES (pH 7.6) and 1 mM EDTA to a protein concentration of 1 mg/ml. They were denatured by addition of solid urea to 8 M and incubated for 4 hr at 21°C. Equal amounts of each protein in 8 M urea were diluted to successively lower concentrations of urea (7, 6, 5, 4, 3, 2, 1 M) buffered with 50 mM HEPES (pH 7.6) and 1 mM EDTA and incubated at room temperature for 2 hr. The samples were centrifuged at full speed in a microfuge. The pellet and supernatant for each urea concentration were analyzed by SDS–PAGE, followed by Coomassie blue staining, to determine if the proteins remained soluble or were aggregating at intermediate concentrations of urea.

2.8. Amylose Resin Binding of Refolded MBP–Aspartic Proteinase Fusions

Ten µg each of refolded MBP–aspartic proteinase fusion proteins was mixed with 25µl of washed amylose resin (New England Biolabs, Beverly, MA) in a 1.8-ml microfuge tube and incubated on ice for 1 hr. The tube was centrifuged for 1 min and the supernatant saved as unbound fraction. The resin was washed with 3 × 1-ml aliquots of 20 mM Tris-Cl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 0.02% Tween-80 buffer by resuspension and centrifugation. Bound protein was eluted with 100µl of the above buffer containing 10 mM maltose, and the unbound and bound fractions were analyzed by SDS–PAGE.

3. RESULTS

3.1. Folded State of Aspartic Proteinase Portions of Fusion Proteins

We previously reported that three aspartic proteinases, procathepsin D, pepsinogen, and prorenin, when expressed as fusions with bacterial MBP at the N-terminus accumulated as soluble proteins in the cytoplasm of *E. coli* even when the propeptides of the aspartic proteinases were deleted (Sachdev and Chirgwin, 1998*b*). These proteins, when expressed unfused, accumulate as insoluble inclusion bodies in *E. coli*. Although the fusions to MBP solved the problem of insolubility, they did not yield active enzymes. Therefore, we analyzed the protein folding states of several of the fusions.

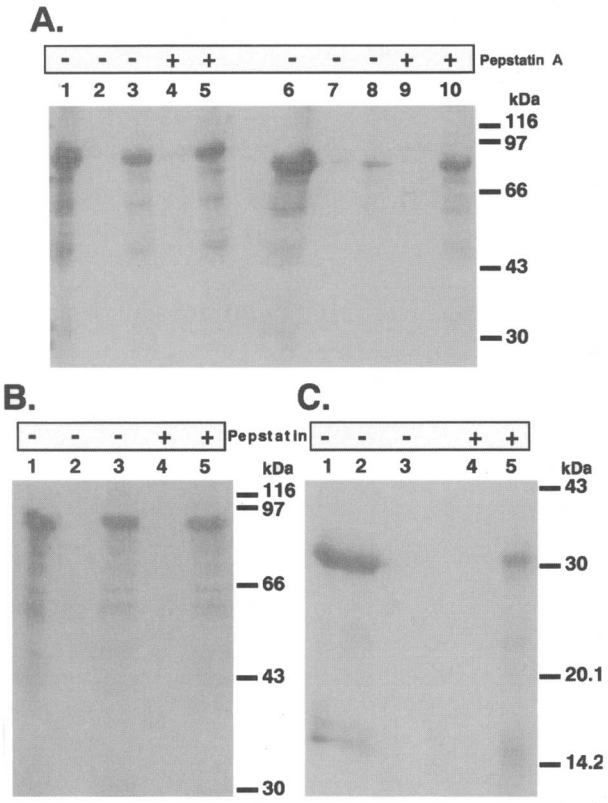

Fig. 1. Pepstatinyl agarose binding of MBP–aspartic proteinases. Fifty μg of each protein was mixed with pepstatinyl agarose for 1 hr at 4°C. The unbound supernatants and bound fractions were analyzed by SDS–PAGE on 10% gels and visualized by Coomassie blue staining. Assays were also done in the presence of soluble 1 μM pepstatin A added to the samples before incubation with pepstatinyl agarose as controls to ensure specific binding. (A) MBP–procathepsin D and MBP–cathepsin D. (B) MBP–pepsinogen. (C) Placental cathepsin D. Lanes 1 and 6 are starting materials used in the assay, lanes 2 and 7 are bound fractions, lanes 3 and 8 are the unbound fractions, lanes 4 and 9 are bound fractions in the presence of soluble pepstatin A added to the samples before incubation with pepstatinyl agarose, and lanes 5 and 10 are the unbound fractions in the presence of added soluble pepstatin A. In panel C only the heavy chain of two-chain mature cathepsin D is visible on the gel.

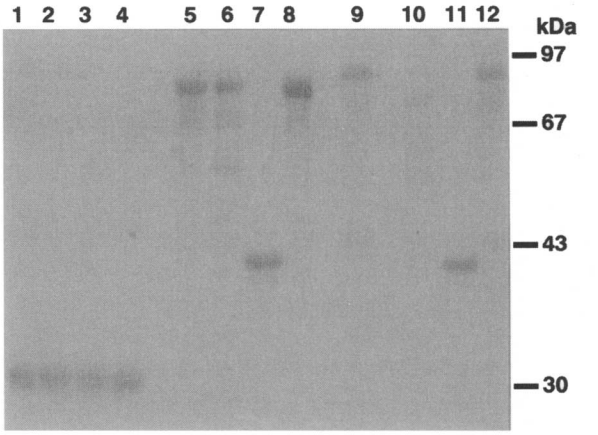

Fig. 2. Proteinase K susceptibility of *E. coli*-expressed MBP–aspartic proteinases. MBP–procathepsin D, MBP–pepsinogen, and placental cathepsin D were treated with 0.1 or 1% (w/w) proteinase K for 1 hr at room temperature. After proteinase K treatment, samples were boiled with Laemmli sample buffer and analyzed by SDS–PAGE followed by Coomassie blue staining. Lanes 1–4: placental cathepsin D; lanes 5–8: MBP–procathepsin D; lanes 9–12: MBP–pepsinogen. Lanes 1, 5, and 9 show samples with no proteinase K treatment. Lanes 2, 6, and 10 were treated for 1 hr with 0.1% proteinase K. Lanes 3, 7, and 11: 1 hr treatment with 1.0% proteinase K. Lanes 4, 8, and 12 are control reactions of the three proteins added to inactivated proteinase K.

We first tested whether the aspartic proteinase moieties were native as judged by binding to immobilized pepstatin. Pepstatin is a transition state analog for aspartic proteinases (Marciniszyn *et al.*, 1976) and binds to the properly folded active site clefts of these enzymes. Mature cathepsin D can bind to pepstatin at pH 5.3 or 3.5, but procathepsin D binds only at pH 3.5 (Conner, 1989). Therefore, 50 μg of purified fusion protein was mixed with pepstatinyl agarose at pH 3.5. The supernatant after centrifugation was saved as the unbound fraction. The bound fraction was eluted with high-pH buffer. The unbound and bound fractions were concentrated by acetone precipitation and analyzed by SDS–PAGE. All of the assays were also performed after preincubating the samples with 1 μM soluble pepstatin A before mixing with resin. This ensured that any binding was specific. Neither MBP–procathepsin D nor MBP–cathepsin D bound significantly to pepstatinyl agarose at pH 3.5 (Fig. 1A). Lanes 1 and 6 are the starting materials used in the assay. MBP–procathepsin D appeared in the pepstatinyl agarose-unbound fraction (lane 3). No protein was in the bound fraction in lane 2. MBP–cathepsin D also did not bind to pepstatinyl agarose (lane 7). Mature cathepsin D isolated from human placentas was included as a positive binding control (Fig. 1C, lane 2). MBP–pepsinogen also failed to bind pepstatinyl agarose (Fig. 1B, lane 2). MBP–procathepsin D, MBP–cathepsin D, and MBP–pepsinogen all remained stable and soluble even at very high concentrations (not shown), indicating they were not grossly aggregated.

The conformations of the various fusion proteins were probed using susceptibility to proteinase K digestion. MBP–procathepsin D and MBP–pepsinogen were treated with 0.1% and 1% proteinase K for 1 hr and the results analyzed by SDS–PAGE (Fig. 2). Purified pla-

cental cathepsin D was the control (lanes 1–4) and was resistant to both 0.1% (lane 2) and 1% (lane 3) proteinase K. Lanes 5–8 are MBP–procathepsin D and lanes 9–12 are MBP–pepsinogen. Lanes 1, 5, and 9 are samples of the various proteins with no proteinase K treatment. Control reactions of the three proteins added to inactivated proteinase K showed no proteolysis (compare lanes 4 vs. 1 for the 31—kDa heavy chain of placental cathepsin D, lanes 8 vs. 5 for MBP–procathepsin D, and lanes 12 vs. 9 for MBP–pepsinogen) indicating that the proteinase K inactivation at the end of the incubations was complete. MBP–pepsinogen was digested to a lower molecular weight species (the size expected for the MBP partner) by 1% proteinase K (lane 11). MBP–procathepsin D was partially resistant to 0.1% proteinase K (lane 6). However, it was digested to a lower molecular weight species by 1% proteinase K (lane 7), corresponding to the size expected for MBP.

We next examined whether one of the fusion proteins displayed increased solvent exposure of hydrophobic residues compared to its unfused component proteins. bis-ANS binds to hydrophobic surfaces, and its fluorescence spectrum can be used to probe the hydrophobic surface exposure of a protein. In Fig. 3, 1 mg/ml each of MBP–cathepsin D, placental cathepsin D, or MBP was mixed with 10 μM bis-ANS and the fluorescence spectra recorded. The binding of bis-ANS to MBP–cathepsin D (filled diamonds) was compared to its binding to MBP (open squares) and to placental cathepsin D (filled circles). If the folded state of the fusion protein were a composite of the folded states of the two individual fusion partners, the fluorescence spectrum of bis-ANS binding to MBP–cathepsin D would be the average of the spectra of placental cathepsin D and MBP; i.e., the spectrum for MBP–cathepsin D would be between that of placental cathepsin D and MBP. However, the fusion protein MBP–cathepsin D, even at half the molar concentration of unfused MBP and placental cathepsin D, showed greater bis-ANS fluorescence, indicating substantially increased hydrophobic exposure. Since a sufficient quantity of native procathepsin D was unavailable, we analyzed only MBP–cathepsin D, and not MBP–procathepsin D, in order to make a direct comparison between bis-ANS binding to a fusion protein and to its individual components.

3.2. Hydrodynamic Properties of Purified MBP Fusions

Sedimentation velocity analysis of the MBP–aspartic proteinases was undertaken to characterize physically the soluble fusion proteins. The sedimentation velocity runs are shown as the integral distribution of sedimentation values ($s_{20,w}$) for the protein. A single pure-component system showing ideal behavior will appear as a vertical line intercepting the x axis at the appropriate $s_{20,w}$ value. Sedimentation velocity analysis of the soluble MBP–aspartic proteinases MBP–procathepsin D, MBP–cathepsin D, and MBP–pepsinogen revealed that all of them were large species with $s_{20,w}$ values greater than 50S. van Holde and Weischet analysis also indicated that they were heterogeneous in size distribution. Figure 4A shows the analysis of MBP (filled squares), which is 42 kDa and can exist as a monomer or a dimer, and placental cathepsin D (open triangles) of ~45 kDa; both showed a homogeneous distribution of species. Figure 4B shows the distribution of $s_{20,w}$ for MBP–procathepsin D (filled squares) and MBP–cathepsin D (open triangles), both of which show a heterogeneous population of species between 50S and 125S. Figure 4C shows the distribution for MBP–pepsinogen, which demonstrates $s_{20,w}$ values of 50–140S. The data indicate that these large, multimeric species were not in dynamic equilibrium with smaller species, such as monomers.

3.3. Properties of Fusion Proteins after Refolding *In Vitro*

Since several aspartic pro-proteinases, such as pepsinogen, are efficiently renatured from urea-denatured inclusion bodies, we wondered whether a cycle of denaturation and refolding would alter the properties of the fusion proteins described above. We asked if the conformation reached by refolding *in vitro* was the same as the folded state assumed by the MBP–aspartic proteinases in the bacterial cytosol, or if denaturation followed by refolding *in vitro* would yield properly folded MBP–aspartic proteinases. The fusion proteins were denatured by incubation with 8 M urea for 4 hr at room temperature and refolded *in vitro* by dialysis to remove the urea, following a protocol for procathepsin B (Kuhelj *et al.*, 1995). All of the MBP–aspartic proteinases remained soluble. After refolding, the proteins were mixed with a 50% v/v slurry of amylose resin in a microfuge tube for 1 hr and the bound and unbound fractions analyzed by SDS–PAGE, as shown in Fig. 5. Solubly expressed MBP bound amylose resin (lane 2). Lane 3 shows the amount of refolded MBP used in the assay. MBP, after denaturation and refolding *in vitro*, bound amylose resin (lane 5), indicating that it has folded into its native conformation. MBP–procathepsin D expressed in *E. coli* also bound amylose resin (lane 7). The amount of re-

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