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Interactions of liver Grp78 and *Escherichia coli* recombinant Grp78 with ATP: Multiple species and disaggregation

(glucose-regulated protein/stress protein)

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ABSTRACT **The hamster gene encoding the 78-kDa glucose-regulated protein (Grp78) was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase. After induction with isopropyl β-D-thiogalactopyranoside, the recombinant Grp78 was purified to homogeneity by affinity column chromatography of the fusion protein followed by thrombin cleavage. The purified recombinant protein was compared with liver Grp78 for its ability to interact with ATP. Like liver Grp78, the recombinant protein contained a weak ATPase activity and a Ca^{2+}-stimulated autophosphorylation activity. However, unlike liver Grp78, in which the autophosphorylation reaction is stimulated <50% by $CaCl_2$, the reaction with the recombinant Grp78 was stimulated about 15-fold in the presence of Ca^{2+}. Although the liver protein showed at least four isoforms after two-dimensional gel electrophoresis, the recombinant Grp78 had one major species corresponding to the most basic form seen in liver. Both the liver Grp78 and the recombinant protein existed primarily as monomers and dimers. A small amount of oligomers was also present in the liver Grp78. When either protein was incubated with ATP, there was a conversion of the higher molecular weight species to the monomeric form.**

The processing of both secretory and nonsecretory proteins in the endoplasmic reticulum (ER) involves a 78-kDa glucose-regulated resident luminal protein (Grp78) (1–3). This protein was also shown to form a stable complex with the heavy chain of immunoglobulin in the ER (4, 5). It appears that Grp78 interacts transiently with normal proteins entering the ER (5–9) but binds strongly to those proteins that are misfolded due to mutations (6) or underglycosylation resulting from glucose starvation or the use of inhibitors of N-linked glycosylation (10, 11). This stable binding results in the retention of the protein in the ER (6) and induction of Grp78 synthesis (6). Grp78 synthesis is also induced by a variety of stress conditions such as viral infection, anoxia, sulfhydryl reagents, and calcium ionophores (reviewed in ref. 12).

Grp78 shares about 60% amino acid homology with the 70-kDa heat shock proteins (Hsp70s) (13) and has several similar biochemical characteristics. Thus both proteins form complexes with target proteins that dissociate in the presence of ATP (13–15). Grp78, like other members of the Hsp70 family, binds to ATP and has a weak ATPase activity (16, 17). In addition Grp78 has been found to be phosphorylated as well as ADP-ribosylated (18–23). We have shown that Grp78 can be autophosphorylated *in vitro* and that this reaction is stimulated by Ca^{2+} (23). Although it is not clear whether phosphorylation affects the function of Grp78, it is known that only the unmodified form of Grp78 is found stably bound to target proteins (19). Other studies have shown that induction of Grp78 synthesis leads to a decrease in the level of phosphorylated Grp78 (23).

In order to better study the structure, posttranslational modifications, and function of Grp78, large amounts of the unmodified protein are required. Although a recombinant protein consisting of Grp78 fused to a portion of the λcII protein has been reported (24), this fusion protein was significantly larger than mature Grp78, owing to the presence of the signal peptide and residues from λcII. Here we describe the overexpression of hamster Grp78 in *Escherichia coli* as a fusion protein containing three additional N-terminal amino acids and compare the purified recombinant Grp78 (rGrp78) and liver Grp78 with respect to their ability to interact with ATP.

MATERIALS AND METHODS

Plasmid pGEX-2T, which contains the glutathione *S*-transferase gene, and glutathione-agarose were purchased from Pharmacia and Sigma, respectively. [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham, and *E. coli* XL1-Blue (25) was from Stratagene. A hamster cDNA Grp78 clone, p3C5 (26), was kindly provided by A. Lee (University of Southern California). Restriction enzymes were obtained from commercial sources and used according to manufacturers' recommendations. Ampholines, thrombin, and gel filtration molecular size standards were purchased from Sigma.

Construction of Glutathione *S*-Transferase–Grp78 Fusion Plasmid. Hamster Grp78 was expressed in *E. coli* using the glutathione *S*-transferase gene fusion system (27). Four overlapping oligodeoxynucleotides (Fig. 1*A*) coding for the first 31 amino acids of the mature form of Grp78 (26) were synthesized on an Applied Biosystems oligonucleotide synthesizer. The most 5′ and 3′ oligonucleotides included a *Bam*HI and a *Hin*dIII site, respectively. The oligonucleotides were annealed at 65°C for 5 min, cooled slowly, and filled in by use of the Klenow fragment of DNA polymerase I. The product was digested with *Bam*HI and *Hin*dIII and ligated to pUC19 that had been similarly digested. The resulting plasmid, pUC19-Grp78′, contained the first 90 nucleotides of the coding sequence of mature Grp78. p3C5 was digested with *Eag* I and *Hin*dIII, and the fragment containing the remaining 3′ nucleotides of the Grp78 sequence was ligated to pUC19-Grp78′ that had been similarly digested. To create a fusion with the glutathione *S*-transferase gene, the resulting plasmid, which contained the complete coding sequence for Grp78, was cleaved with *Bam*HI and *Ssp* I and ligated to pGEX-2T that had been digested with *Bam*HI and *Sma* I. The resulting plasmid, pDS78, which bears the coding sequence

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Abbreviations: ER, endoplasmic reticulum; Grp, glucose-regulated protein; rGRP, recombinant GRP; Hsp, heat shock protein; IPTG, isopropyl β-D-thiogalactopyranoside.

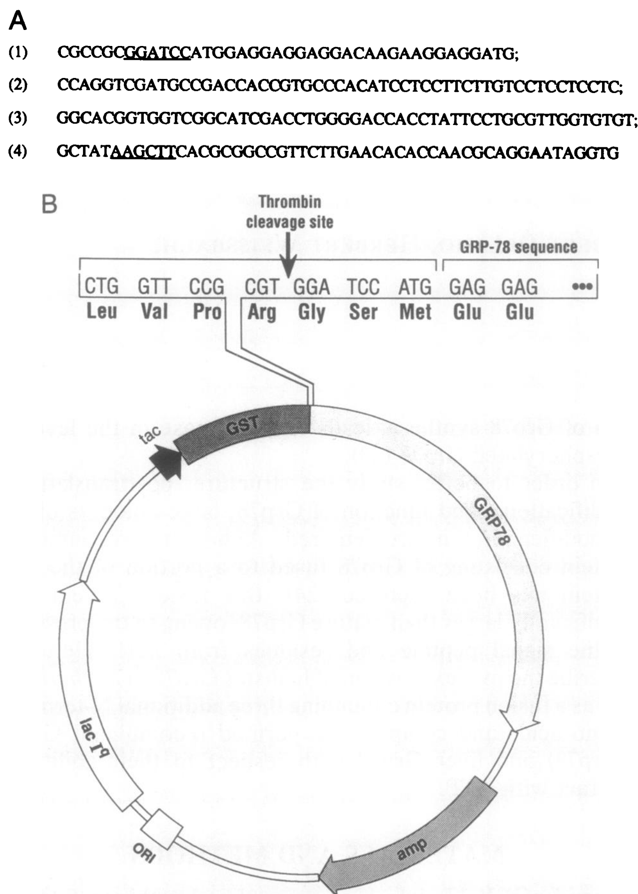

FIG. 1. Glutathione *S*-transferase–Grp78 fusion plasmid. (*A*) Four overlapping oligonucleotides spanning the first 90 nucleotides of the mature protein (24) were used to reconstruct the 5′ end of the hamster Grp78 gene. The first and fourth oligonucleotides contain a *Bam*HI and *Hin*dIII site, respectively (underlined). (*B*) The modified gene was then cloned into pGEX-2T, in frame with the glutathione *S*-transferase gene (GST), giving plasmid pDS78. The figure shows the thrombin cleavage site and the N-terminal amino acids of the recombinant protein after cleavage of the fusion protein with thrombin.

for hamster Grp78 in frame with the glutathione *S*-transferase gene, was transformed into *E. coli* XL1-Blue.

Purification of rGrp78. *E. coli* XL1-Blue/pDS78 was grown at 37°C in LB medium containing ampicillin (50 μg/ml). When the cells had reached an OD_{600} of 1.0, isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM) was added to the medium and the culture was incubated for 3 hr. In a typical purification, 2.5 g of cells were resuspended in 15 ml of 130 mM NaCl/20 mM $NaHPO_4$, pH 7.4, containing 1% (vol/vol) Triton X-100 and were broken by sonication. The lysate was centrifuged at 10,000 × *g* for 7 min, and the supernatant (14 ml, 256 mg of protein) was mixed at 4°C with 10 ml of glutathione-agarose that had been equilibrated with the above buffer. The resin was allowed to settle, the supernatant was removed, and the resin was washed with 10 volumes of buffer A (50 mM Tris Cl, pH 7.4/150 mM NaCl). After settling the resin was suspended in 5 ml of buffer A containing 5 mM $CaCl_2$ and 200 units of thrombin. The suspension was mixed for 1–2 hr at 4°C and, after settling, the supernatant was removed and the agarose was washed with 10 ml of buffer A and allowed to settle. This supernatant was removed, combined with the first supernatant obtained after the thrombin treatment, and dialyzed against buffer B (20 mM Tris Cl, pH 7.4/20 mM KCl/2 mM $MgCl_2$). In some instances the protein was further purified by DEAE-cellulose chromatography as follows. The protein eluted from the glutathione-agarose was dialyzed against 50 mM Tris Cl, pH 7.5/50 mM NaCl and loaded onto a DEAE-cellulose column that had been equilibrated with this buffer. The protein was then eluted with a gradient of 50–300 mM NaCl in 50 mM Tris Cl, pH 7.5.

Purification of Bovine and Hamster Liver Grp78. Grp78 was purified from bovine and hamster liver microsomes by DEAE-cellulose and ATP-agarose chromatography as previously described, except that EDTA was omitted and the ATP was removed by ultrafiltration or $(NH_4)_2SO_4$ precipitation and dialysis (23, 28, 29). Unless otherwise mentioned, bovine liver Grp78 was routinely used in these studies.

Autophosphorylation and ATP Hydrolysis Assays. The incubation conditions for *in vitro* phosphorylation of Grp78 with [γ-^{32}P]ATP have been described (23). To quantitate the phosphorylation, an aliquot of the reaction mixture was spotted onto a nitrocellulose filter and soaked for 15 min in cold 10% Cl_3CCOOH containing 2 mM ATP. The filters were washed three times in this solution and suspended in scintillation fluid for measurement of radioactivity. The incubation mixture for the hydrolysis of ATP contained, in a total volume of 20 μl, 20 mM Mes/NaOH (pH 6.0), 1 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (1 μCi), and various amounts of Grp78. The mixture was incubated for 10 min at 37°C and the amount of orthophosphate liberated was determined by the method of Conway and Lipmann (30). Details of the two-dimensional gel analysis and the separation of monomer and aggregate forms of Grp78 are described in the legends to Figs. 5 and 6, respectively.

RESULTS

Expression and Purification of rGrp78. To obtain large amounts of unmodified Grp78 for structure–function studies, we expressed hamster Grp78 in *E. coli*. The Grp78 gene was ligated into a vector in frame with an IPTG-inducible glutathione *S*-transferase gene containing a thrombin cleavage site. The resulting fusion protein could then be cleaved with thrombin, generating Grp78 containing three additional N-terminal amino acids (Fig. 1*B*).

The plasmid described above, pDS78, was transformed into *E. coli* XL1-Blue, the synthesis of the fusion protein was induced with IPTG, and the protein was purified as described in *Materials and Methods*. A protein of about 105 kDa was present in crude cell-free extracts only after induction with IPTG (Fig. 2, lane 2 vs. lane 1). This molecular mass is consistent with a fusion protein containing Grp78 (78 kDa) and glutathione *S*-transferase (27 kDa). After centrifugation at 10,000 × *g* for 7 min, very little of the fusion protein was found in the pellet, the bulk being present in the S-10 extract (lane 3). The fusion protein was bound to glutathione-agarose, and after treatment with thrombin the Grp78 migrated at ≈78 kDa (lane 4), similar to liver Grp78 (data not shown). In some cases the protein was further purified by DEAE-chromatography (lane 5). The Grp78 in this fraction was estimated to be 95% pure. Approximately 8.4 mg of purified protein (after the affinity chromatography step) was obtained from 2.5 g of cells.

Autophosphorylation and ATPase Activity of rGrp78. Liver Grp78 can be autophosphorylated, and this reaction is slightly stimulated by Ca^{2+} (23). In addition, the protein contains an ATPase activity (16, 17). Fig. 3 compares the autophosphorylation activity of rGRP78 and liver Grp78. Although the reaction with liver Grp78 was stimulated <2-fold upon addition of Ca^{2+}, the autophosphorylation of the recombinant protein was stimulated about 15-fold. However, even at the optimum Ca^{2+} concentration (100 μM), the autophosphorylation of rGrp78 was about 20% that of the liver Grp78. A similar ratio of activities was observed in the

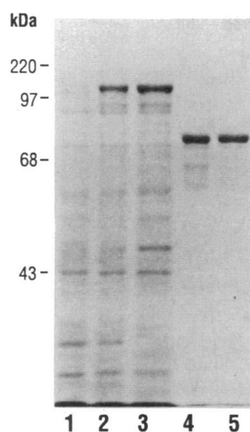

FIG. 2. SDS/PAGE of fractions during purification of rGrp78. *E. coli* XL1-Blue/pDS78 was grown to an OD_{600} of 1, an aliquot of the cells was removed, and the remaining cells were incubated with IPTG for 3 hr. The cells were harvested and the Grp78 was purified as described in the text. Lane 1, uninduced crude lysate; lane 2, induced crude lysate; lane 3, induced S-10 fraction; lane 4, glutathione-agarose eluate after thrombin treatment; lane 5, DEAE-cellulose fraction. Ten micrograms of protein was applied to each lane, and the gels were stained with Coomassie blue.

case of ATP hydrolysis, with the recombinant protein about 30% as active as the liver protein (Fig. 4).

Isoforms of rGrp78. Grp78 isolated from tissues is both phosphorylated and ADP-ribosylated (18–22). The function of these posttranslational modifications is unknown and one of the primary reasons for obtaining rGrp78 was to allow structure–function studies of an unmodified form of the protein. Since the various isoforms of the protein can be separated by isoelectric focusing (23), it was important to determine whether rGrp78 was unmodified compared with Grp78 purified from bovine liver. Four isoforms of the Grp78 were isolated from beef liver (Fig. 5*A*), similar to the pattern observed previously with Grp78 isolated from MDBK cells (23). However, only one major isoform was observed in the rGrp78 preparation (Fig. 5*C*), which migrated with the most basic form of liver Grp78. This suggests that rGrp78 is neither phosphorylated nor ADP-ribosylated. When these proteins were incubated in the presence of [γ-^{32}P]ATP and Ca^{2+}, as described above in Fig. 3, the three more acidic isoforms of the liver Grp78 were labeled (Fig. 5*B*), and the labeled rGrp78 migrated to a more acidic position than the unlabeled protein

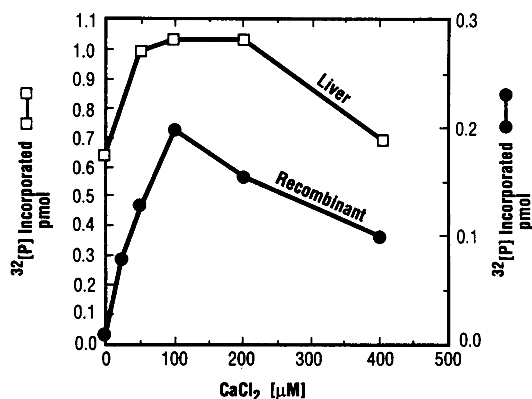

FIG. 3. Effect of Ca^{2+} on the autophosphorylation of liver Grp78 and rGrp78. The reaction mixture for the autophosphorylation reaction has been described (23). In these assays, 2.5 μg (35 pmol) of either liver Grp78 or rGrp78 was used. Various concentrations of Ca^{2+} were added as indicated. The reaction mixture was incubated for 60 min at 37°C.

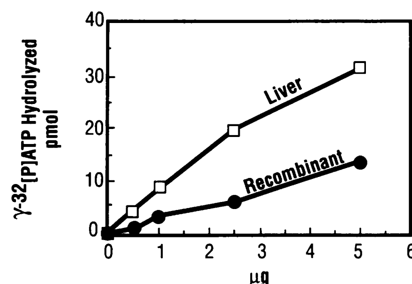

FIG. 4. Effect of Grp78 concentration on the hydrolysis of ATP. Various amounts of purified liver Grp78 or rGrp78 were incubated for 10 min at 37°C and assayed for the amount of orthophosphate released (30).

(Fig. 5*D*). Phospho amino acid analysis (23) showed that threonine was the phosphorylated amino acid in both bovine liver Grp78 and rGrp78 (data not shown). The experiments described in Fig. 5 *A* and *C* were performed with bovine liver Grp78, but similar results were obtained with hamster liver Grp78.

Aggregate Forms of Grp78 and Dissociation by ATP. rGrp78 was also compared with liver Grp78 for the presence of aggregate forms of the protein. Results of a typical experiment are shown in Fig. 6. As assayed by HPLC gel filtration, liver Grp78 (Fig. 6*A*) contained a mixture of monomer and dimer forms as well as a small amount of heavier species (oligomers). The amount of the oligomeric forms varied in different preparations, but all preparations contained significant amounts of dimer and monomer. rGrp78 also existed as a mixture of monomers and dimers (Fig. 6*B*). A striking effect on the Grp78 aggregates was observed after incubation of the protein with ATP at 0°C. There was a decrease in the dimer and oligomer forms of liver Grp78, with a concomitant increase in the monomer form, in the presence of ATP (Fig. 6*C*). Similarly, the dimer peak of rGrp78 was also significantly reduced after incubation with ATP, with an increase in the monomer peak (Fig. 6*D*).

DISCUSSION

In this report we compare the characteristics of purified rGrp78 isolated from *E. coli* with those of purified bovine liver Grp78. The recombinant protein was initially expressed as a fusion protein with glutathione *S*-transferase. After

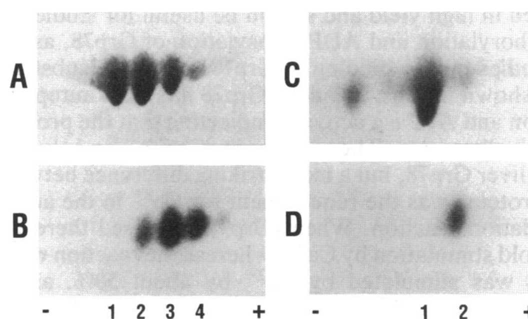

FIG. 5. Two-dimensional polyacrylamide gel analysis of liver Grp78 and rGrp78 phosphorylated *in vitro*. Purified liver Grp78 and rGrp78 (2.5 μg) were phosphorylated *in vitro* and the various isoforms of the proteins were then resolved by two-dimensional gel electrophoresis (23). Gels were run in the first dimension in tubes (0.2 × 14 cm), using pH 4.5–5.4 Ampholines, and in the second dimension in a 7.5% polyacrylamide/SDS gel. The gels were stained with Coomassie blue prior to autoradiography. (*A*) Coomassie-stained liver Grp78. (*B*) Autoradiography of *A*. (*C*) Coomassie-stained rGrp78. (*D*) Autoradiography of *C*. Isoforms are numbered from the most basic, 1, to the most acidic, 4.

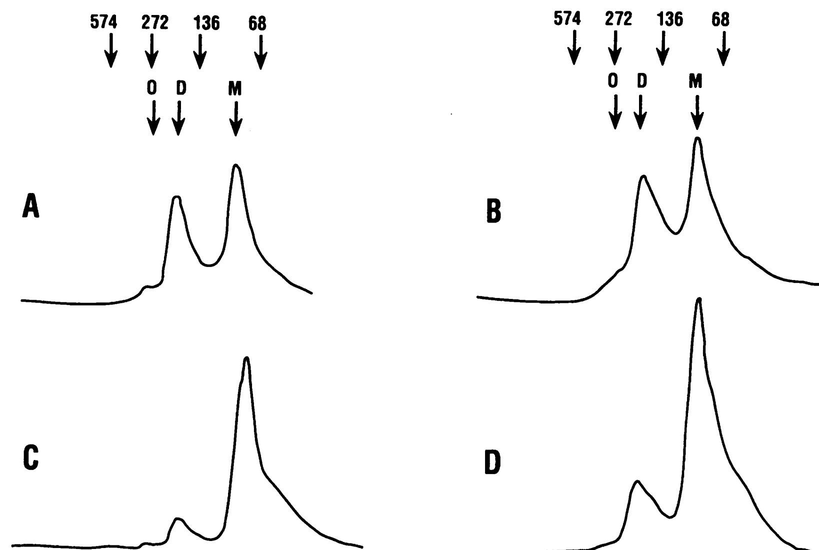

FIG. 6. HPLC gel filtration chromatography of liver Grp78 and rGrp78. About 15 μg of each purified protein was incubated at 0°C for 15 min in a final volume of 50 μl in the absence or presence of 1 mM ATP. The reaction mixtures were then applied to a Bio-Sil SEC-250 HPLC gel filtration column (0.75 × 60 cm, Bio-Rad) that had been equilibrated with a buffer containing 20 mM Tris Cl (pH 7.0) and 200 mM KCl. The protein was eluted from the column with this buffer at a flow rate of 0.3 ml/min. The protein eluted from the column was detected by UV absorption at 215 nm. (*A*) Liver Grp78. (*B*) rGrp78. (*C*) Liver Grp78 plus ATP. (*D*) rGrp78 plus ATP. Peaks: M, monomer; D, dimer; O, oligomer or higher molecular weight aggregates. Molecular size markers were hexameric urease (574 kDa), trimeric urease (272 kDa), dimeric albumin (136 kDa), and monomeric albumin (68 kDa).

glutathione-agarose affinity chromatography, thrombin cleavage, and DEAE-cellulose chromatography, highly purified (>95%) rGrp78 was obtained (Fig. 2). The protein comigrated with bovine and hamster liver Grp78 and reacted with antibody raised against bovine liver Grp78 (data not shown). Although rGrp78 has not been sequenced, it presumably differs from the mature form of hamster liver Grp78 by having an additional three amino acids at the N terminus derived from the construction of the recombinant plasmid (Fig. 1). The rGrp78 protein migrated as a single major species after isoelectric focusing, in contrast to liver Grp78, which showed the presence of three prominent more acidic species. The difference is thought to reflect the fact that the liver protein is both phosphorylated and ADP-ribosylated, whereas rGrp78 is presumably unmodified. After autophosphorylation, the ^{32}P-labeled rGrp78 migrated to a more acidic position, consistent with the idea that the recombinant protein is unmodified. This recombinant protein can be easily purified in high yield and should be useful for studies on the phosphorylation and ADP-ribosylation of Grp78, as well as for studies on the binding of Grp78 to protein substrates.

As shown in Figs. 3 and 4, rGrp78 had both autophosphorylation and ATPase activity, indicating that the protein was biologically active. The activity of rGrp78 was only 20–30% of the liver Grp78, but a most striking difference between the two proteins was the requirement for Ca^{2+} in the autophosphorylation reaction. When rGrp78 was used there was an ≈15-fold stimulation by Ca^{2+}, whereas the reaction with liver Grp78 was stimulated by Ca^{2+} by about 50%, as shown previously (23). The previous results suggested either that purified liver Grp78 contained tightly bound Ca^{2+} or that there was sufficient Ca^{2+} contaminating the reaction components used in the assay (23). The present results support the view that liver Grp78, but not rGrp78, contains tightly bound Ca^{2+}. The ER sequesters the bulk of the cellular Ca^{2+} (31), and it is possible that there is a specific mechanism in the ER to bind Ca^{2+} to the protein. It is interesting that Ca^{2+} has also been implicated in the induction of Grp78 synthesis. When cells are incubated with a Ca^{2+} ionophore, Ca^{2+} is released from the ER into the cytoplasm and there is a large increase in the synthesis of Grp78 (18, 32, 33). The Grp78 isolated under these conditions is unphosphorylated (19, 23) and is a poor substrate for phosphorylation *in vitro* (data not shown).

In addition to the observation that Grp78 exists in various forms due to posttranslational modifications, we have shown that the protein is found as multimers. Both the recombinant and the liver Grp78 protein preparations contained mostly monomers and dimers, and the liver Grp78 also contained higher molecular weight aggregates. Perhaps the most important observation in this study is that the multimeric forms of both liver Grp78 and rGrp78 could be converted in large part to a monomer after incubation with ATP. This result is consonant with the studies of Palleros *et al.* (34), who reported that ATP dissociated trimers and dimers of bovine brain Hsp73, and suggests a common mechanism for Hsp70 self-association and dissociation.

The ability of ATP to disaggregate oligomers of Grp78 may explain differences that have been reported regarding the size of the isolated protein and provide information on how this protein may function. In the original isolation of Grp78, described by Shiu and Pastan (3), the authors noted that the protein had a molecular weight of ≈400,000 by gel filtration. Kassenbrock and Kelly (17) reported that the protein was mostly a monomer, but they used ATP-agarose chromatography in their purification whereas Shiu and Pastan (3) did not. Members of the Hsp70 family are routinely purified by ATP-agarose chromatography (29), as was the liver Grp78 used in this study. All of our liver Grp78 preparations after ATP-agarose purification were further treated by ultrafiltration or $(NH_4)_2SO_4$ precipitation to remove the bulk of the ATP. Before removal of ATP the protein behaves as a monomer, but after the ATP is removed there is clear evidence that dimers are present, showing that the Grp78 monomers and dimers may be interconvertible (data not shown). We have also partially purified liver Grp78 by DEAE chromatography and FPLC, avoiding the ATP-agarose step. This protein also behaved as a complex mixture, containing mostly high molecular weight aggregates with much smaller amounts of dimer and monomer than seen after purification with ATP-agarose. As with the protein purified on ATP-agarose, incubation in the presence of ATP resulted in a

decrease in the amount of heavy aggregates and an increase in the monomeric form (data not shown). Since the bulk of the Grp78 present *in vivo* may be in the form of oligomers, it is possible that high molecular forms of Grp78 are involved in the binding of misfolded proteins. This complex could be dissociated in the presence of ATP with the concomitant formation of the Grp78 monomer and release of the bound protein in a properly folded structure. Therefore it will be essential to compare the various forms of Grp78 to determine which species have chaperone activity. It may be that the Grp78 monomer is an end product of this process and that the ER may possess an efficient mechanism for converting the monomer back to higher molecular weight forms. If so, the interaction of ATP-agarose-purified Grp78 with protein and peptide substrates may not reflect what normally occurs *in vivo*. Experiments are needed to determine the form of Grp78 that interacts with proteins passing through the ER, and the role of Ca^{2+} and ATP in this process.

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