Novel 30 kDa protein possessing ATP-binding and chaperone activities

Hideaki ITOH¹ and Yohtalou TASHIMA

Department of Biochemistry, Akita University School of Medicine, 1-1-1 Hondo, Akita City, 010 Japan

A 30 kDa protein was purified from pig liver cytosol by using ATP-Sepharose and Green A column chromatography. The partial amino acid sequences of the protein (95 amino acid residues) had no similarity with any proteins recorded in data banks. The protein was able to form a stable complex with unfolded dihydrofolate reductase (DHFR). The spontaneous refolding of chemically denatured DHFR was arrested by the 30 kDa protein. This inhibition presumably results from the formation of a stable complex between the 30 kDa protein and DHFR. Bound DHFR could be released from the protein with ATP. The protein also showed protease resistance in an ATP-

dependent manner. Incubation of the 30 kDa protein with 5 mM ATP resulted in its resistance to V_8 protease or to trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one. Divalent cations enhanced the ATP-protection effect. CD analysis of the 30 kDa protein showed that ATP induced an increase in the β -pleated sheet content and a decrease in the α -helix content of the 30 kDa protein. These results suggest that the 30 kDa protein, a novel cytosolic protein, might have an affinity for ATP, a chaperonin activity, and an ATP-protection effect against some proteases *in vivo*.

INTRODUCTION

Cells respond to external stresses such as a sudden increase in temperature with the synthesis of a distinct set of proteins called heat-shock proteins (HSPs) or stress proteins [1]. HSPs are divided into subcategories on the basis of molecular mass ranges or their sequence similarities. Three major classes of HSP are the 70 kDa HSP70 family, the 90 kDa HSP90 family and the low-molecular-mass HSPs including the 60 kDa HSP60 family [2]. The predominant classes of stress protein have been implicated in protein folding as molecular chaperones [3,4].

Molecular chaperones, some of which are also HSPs, participate in the biogenesis of proteins including their synthesis, folding, assembly, disassembly and translation [1,3,5]. Molecular chaperones have the general property of interacting with other proteins in their non-native conformations.

The chaperones of the HSP70 and HSP60 families function by preventing aggregation of newly synthesized polypeptides; they then mediate their folding to the native state in an ATP-dependent process [1]. HSP70 is thought to 'chaperone' protein folding by, at a minimum, binding to a nascent or misfolded segment of polypeptides, thereby inhibiting their aggregation. HSP90 has been implicated as a molecular chaperone in the maturation *in vivo* of specific protein substrates such as steroid receptors and kinase [5]. Assembly of these complexes with substrate proteins has been found to be ATP-dependent [6].

Despite the apparent diversity of these activities, molecular chaperones might serve a common function to influence the conformational state of many proteins. Protein folding by the double-ring chaperonin GroEL is initiated in *cis* ternary complexes, in which polypeptide is sequenced in the central channel of a GroEL ring, capped by the co-chaperonin GroES [7,8]. The *cis* ternary complex is dissociated by *trans*-sided ATP hydrolysis, which triggers the release of GroES [9]. More recently it has been

shown that both native and non-native proteins are released from a *cis*-only GroEL ternary complex [10]. In general, HSPs bind to ATP; binding of ATP was suggested to result in conformational changes in those proteins [11,12].

As well as HSPs, protein disulphide isomerase also acts like a chaperone by binding non-specifically to an exposed polypeptide backbone, the presence of a cysteine residue increasing its affinity [13]. Protein disulphide isomerase has been shown to characterize the isomerization and oxidation of intramolecular disulphide bonds and in so doing to accelerate and promote the correct folding of protein *in vivo* [13]. We recently identified and characterized mammalian HSP60 (chaperonin homologue) [14]. The protein possesses ATP-binding and chaperone activities. During the purification of HSP60 we found another protein in pig liver cytosol that bound to an ATP-Sepharose column. The results of partial amino acid sequences of the protein showed that the protein had no similarity with any other proteins recorded in data banks. Because almost all HSPs have an affinity with ATP and show molecular chaperone activity, we analysed whether or not the liver protein had chaperone activity.

In the present study we have characterized the biochemical properties of the protein, including the influence of ATP, and discuss its possible roles especially as a molecular chaperone.

MATERIALS AND METHODS

Materials

ATP-Sepharose was prepared as previously described [14]. DE-52 and Q-Sepharose were from Pharmacia (Uppsala, Sweden). Gigapite was from Seikagaku Kogyo (Tokyo, Japan) and Matrex Gel Green A was from Amicon (Danvers, MA, U.S.A.). Lysyl endopeptidase (EC 3.4.21.50) was from Wako Pure

Abbreviations used: DHFR, dihydrofolate reductase; HSP, heat-shock protein; HSP90, HSP70 and HSP60, heat-shock proteins with subunit molecular masses of 90, 70 and 60 kDa; Tos-Phe-CH₂Cl, 'tosylphenylalanylchloromethane' (1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one).

¹ To whom correspondence should be addressed.

Chemical Industries (Osaka, Japan). Trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('tosylphenylalanylchloromethane'; Tos-Phe-CH₂Cl) was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). *Staphyrococcus aureus* V₈ protease (EC 3.4.21.19) was from Boehringer Mannheim (Mannheim, Germany). Fresh pig livers were obtained from a local slaughterhouse and stored at -30 °C before use.

Isolation of the 30 kDa ATP-binding protein from pig liver

Purification of the 30 kDa ATP-binding protein involved a modified version of the purification method for HSP60 or HSP70 as previously described [14,15]. All operations were performed at 0–4 °C. Frozen livers (approx. 900 g) were chopped and then homogenized with 3 vol. of buffer A [10 mM Tris/HCl (pH 7.4)/15 mM 2-mercaptoethanol/0.1 % PMSF]. The homogenate was centrifuged at 20000 *g* for 20 min. The supernatant was fractionated with (NH₄)₂SO₄ added at a concentration of 27.7 g/100 ml. After the mixture had been stirred for 30 min the supernatants were collected by centrifugation at 20000 *g* for 10 min, and additional (NH₄)₂SO₄ was added at 33.9 g/100 ml of supernatant. The precipitates were collected by centrifugation, dissolved in buffer B [10 mM Tris/HCl (pH 7.4)/15 mM 2-mercaptoethanol] and dialysed overnight against buffer B; the dialysis solution was changed several times. The dialysate was applied to a DEAE-cellulose column (5 cm × 10 cm) pre-equilibrated with buffer B. After the column had been washed with buffer C (10 mM Tris/HCl, pH 7.4), the proteins were eluted with three column-bed volumes of buffer C containing 0.15 M NaCl. The eluate fractions containing protein peaks were combined and MgCl₂ was added at a final concentration of 5 mM. The solution was applied to an ATP-Sepharose column (2 cm × 7 cm) pre-equilibrated with buffer D [10 mM Tris/HCl (pH 7.4)/5 mM MgCl₂]. The column was washed with three column-bed volumes of buffer D containing 0.5 M NaCl and then washed with the same volume of buffer D. The proteins were eluted with buffer D containing 3 mM ATP and fractions were collected from the column. The eluate fractions containing protein peaks were combined and applied to a Q-Sepharose column (2.5 cm × 5 cm) pre-equilibrated with buffer C. The column was washed with three column-bed volumes of buffer C and eluted with a linear gradient of 0–0.5 M NaCl in buffer C. The 30 kDa protein-rich fractions were combined and dialysed overnight against buffer E (10 mM potassium phosphate, pH 7.0). The dialysate was applied to a Gigapite column (2 cm × 5 cm) pre-equilibrated with buffer E. After the column had been washed with buffer E, the proteins were eluted with a linear gradient of 0.01–0.5 M potassium phosphate, pH 7.0. The 30 kDa protein-rich fractions were combined and dialysed overnight against buffer C. After dialysis the solution was applied to a Green A column (1.5 cm × 4 cm) pre-equilibrated with buffer C. The column was washed with the same buffer and the proteins were eluted with a linear gradient of 0–1.0 M NaCl in buffer C. The protein peak fractions were combined and stored at -80 °C.

Gel electrophoresis

SDS/PAGE was performed by the procedure of Laemmli [16] with 9 % or 12 % (w/v) polyacrylamide gel. After electrophoresis the gels were stained with 0.1 % Coomassie Brilliant Blue (R-250) in 25 % isopropyl alcohol/10 % acetic acid and destained with 10 % isopropyl alcohol/10 % acetic acid.

Gel filtration of the 30 kDa ATP-binding protein

The native molecular mass of the ATP-binding protein was determined by gel filtration as previously described [17].

Amino acid sequence of the 30 kDa liver protein

The purified 30 kDa liver protein or the 78, 73, 60 and 43 kDa proteins eluted from the ATP-Sepharose column were subjected to SDS/PAGE, stained with 0.1 % Coomassie Brilliant Blue (R-250) in 25 % isopropyl alcohol, and the protein bands excised. By using a lysyl endopeptidase, the production and separation of peptides from the protein were performed by the method of Kawasaki et al. [18]. The peptides were purified by HPLC. A column of Wakopak (Wakosil 5C₁₈) was connected to an HPLC apparatus (a Pharmacia LKB HPLC equipped with two Model 2150 pumps, a Model 2150 HPLC controller and a Model 2158 Uvicord SD UV detector was used for purification of peptides). The peptide was applied to the column pre-equilibrated with 0.1 % trifluoroacetic acid and eluted with a linear gradient of 0–60 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected. The purified peptides were detected at 206 nm. The determination of the N-terminal amino acid sequence of the purified peptide was performed with a model 477A protein sequencer with an on-line 120A phenylthiohydantoin analyser (Applied Biosystems, Foster City, CA, U.S.A.). The amino acid sequences obtained for the 30 kDa protein or the 78, 73, 60 and 43 kDa proteins were searched for in the NBRF-PIR or SWISS-PROT data bank.

Spectrophotometric assay of dihydrofolate reductase (DHFR)

For spectrophotometric assays of DHFR, unfolded DHFR was prepared by denaturing native DHFR in 5 M guanidine hydrochloride/0.1 M Tris/HCl (pH 7.4)/1 mM EDTA at a final concentration of 20 μM. To ensure rapid mixing during experiments, a small amount (5 μl) of denatured or native DHFR was added as a drop to the inside of the cap of a Microfuge tube, the bottom of which contained the other reaction ingredients. After the cap was replaced, the reaction was initiated by rapidly inverting the tube and vigorously mixing its contents. DHFR enzyme assays were conducted in quartz cuvettes with a 1 cm path length by the method of Viitanen et al. [19]. Enzyme activity was assayed in the direction of NADPH oxidation in the presence of dihydrofolate. Oxidation of NADPH was monitored at 340 nm with a Hitachi 220A spectrophotometer. The reactions (final volume 600 μl) were set up in 1.5 ml Microfuge tubes containing 0.05 M Tris/HCl, pH 7.4, 5 mM MgCl₂, 3.3 mM KCl and 10 mM dithiothreitol in the presence or absence of the purified 30 kDa protein (1.0 μM). The reaction was initiated by the addition of denatured DHFR to a final concentration of 0.5 μM. After a 2 or 4 h incubation period all reactions were supplemented with dihydrofolate (0.1 mM) and NADPH (0.1 mM). Other reactions were conducted in a solution containing 50 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 3.3 mM KCl, 10 mM dithiothreitol, 100 μM dihydrofolate and 75 μM NADPH in the presence or absence of the 30 kDa protein. Reaction mixtures were then immediately transferred to cuvettes and *A*₃₄₀ was monitored. Values plotted in figures (e.g. Δ*A*₃₄₀) were arbitrarily normalized to a starting *A*₃₄₀ value of 1.0. Additional experimental details are shown in the figure legends.

ATPase activity assay

Assay of the ATPase activity of the purified 30 kDa protein was performed by the procedure of Mendoza et al. [20]. The 30 kDa protein (5 μM) was incubated in a buffer containing 50 mM

Tris/HCl, pH 7.4, 10 mM MgCl_2 and 10 mM KCl at 37 °C for 5 min. After incubation, ATP (1 mM) was added to the incubation mixture. Periodically, 0.12 ml aliquots were removed and mixed with 3 ml of the 260-3 diagnostic reagent, (Sigma), which forms a complex with phosphate released after ATP hydrolysis; the absorbance at 340 nm was measured with Hitachi 220A spectrophotometer.

Digestion of the 30 kDa ATP-binding protein with trypsin or V_8 protease

The purified 30 kDa ATP-binding protein (0.1 mg/ml) was digested with V_8 protease or Tos-Phe- CH_2Cl -treated trypsin (protein-to-enzyme ratio 500:1) in the presence or absence of 5 mM MgCl_2 /5 mM CaCl_2 /5 mM ATP in 10 mM Tris/HCl (pH 7.4) for 30 min at 37 °C. After digestion the samples were subjected to SDS/PAGE.

CD spectroscopy

CD measurements were performed on a Jasco J-720 spectropolarimeter calibrated with *dextro*-10-camphorsulphonic acid. Spectra were recorded in 50 mM Hepes buffer, pH 7.4, at 24 °C in a cylindrical quartz cuvette with a 0.01 cm path length. The protein concentration was kept at 0.1 mg/ml in these experiments. Spectra were the average of four scans at 50 nm/min, each with the bandwidth set at 2 nm, and were baseline-corrected by subtracting the corresponding blank. Spectra were recorded in the range 195–240 nm. The observed ellipticity was converted to mean residue ellipticity $\{[\theta]_{\text{m.r.w.}} \text{ (degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1})\}$. Smoothing was applied to the curves, by a mild function that increased the signal-to-noise ratio without altering the shape of spectra, with the Jasco J-720 program.

RESULTS

Purification of the 30 kDa ATP-binding protein

The 30 kDa ATP-binding protein was purified by the method described in the Materials and methods section. Among the proteins (subunit molecular masses 78, 73, 68, 60, 43 and 30 kDa) that were eluted from the ATP-Sepharose column, the 30 kDa protein was one of the major components (Figure 1). After SDS/PAGE [9% (w/v) gel], these proteins were excised and digested with lysyl endopeptidase; digests were purified by HPLC and subsequently sequenced by a protein sequencer. On the basis of sequence similarity, the 78, 73, 68, 60 and 43 kDa liver proteins were concluded to be identical with GRP78, HSP73, the N-terminal fragment of HSP73, HSP60 and the N-terminal fragment of HSP73 respectively (results not shown). The 30 kDa protein was unknown and was further purified by using Q-Sepharose column chromatography, Gigapite column chromatography and Green A column chromatography. Only the 30 kDa protein was eluted from the Green A column (Figure 2); 5 mg of the protein was obtained from 900 g of fresh pig liver.

Amino acid sequence of the 30 kDa liver protein

The protein was partly digested with lysyl endopeptidase, and the peptides were purified by HPLC with a reverse-phase column (Figure 3A). The nine peptides (nos. 26, 30, 41, 48, 50, 52, 60, 62 and 88) and the N-terminus of the protein were sequenced by a protein sequencer (Figure 3B). Among the nine peptides, nos. 48 and 62 gave similar sequences. The total of 95 amino acid sequences from the nine peptides and the N-terminus of the protein had no similarity to sequences of any proteins registered

Figure 1 ATP-Sepharose column chromatography of pig liver proteins

The eluted fractions from the DEAE-cellulose column were chromatographed on an ATP-Sepharose column and the proteins were eluted with 3 mM ATP. (A) Elution pattern of protein from the ATP-Sepharose column. (B) The eluted fractions were subjected to SDS/PAGE [9% (w/v) gel] followed by Coomassie Brilliant Blue staining. Lane numbers correspond to the fraction numbers. Lane M, standard marker proteins: phosphorylase *b* (94 kDa), BSA (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

Figure 2 Green A column chromatography of the 30 kDa protein

The eluted fractions from the hydroxyapatite column were chromatographed on a Green A column with a linear gradient of 0–1.0 M NaCl. (A) Elution pattern of protein. (B) The fractions (fraction numbers 11–19) were analysed by SDS/PAGE [9% (w/v) gel] with Coomassie Brilliant Blue staining. Lanes 0 and M indicate the eluted fractions of the hydroxyapatite column and standard marker proteins respectively.

Figure 3 HPLC fractionation of lysyl endopeptidase digests and their amino acid sequence data for the 30 kDa liver protein

(A) Lysyl endopeptidase digests of the 30 kDa liver protein were separated by reverse-phase chromatography on a C₁₈ column with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. The purified peptides indicated in the panel (nos. 26, 30, 41, 48, 50, 52, 60, 62 and 88) were sequenced by a peptide sequencer. (B) The nine purified peptides of the lysyl endopeptidase digests (nos. 26, 30, 41, 48, 50, 52, 60, 62 and 88) and the N-terminus of the 30 kDa protein were sequenced. X indicates that the amino acid residue could not be determined.

in the SWISS-PROT or NBRF-PIR data banks. Thus the 30 kDa protein is a newly found one.

Characterization of the 30 kDa liver protein

To determine the native molecular mass of the 30 kDa protein, gel filtration was performed. The protein was eluted 18.8 min after injection on HPLC with a G3000SW column (Figure 4). The molecular mass of the protein was calculated to be 30 000 Da, indicating that the protein is a monomer. The ability of the 30 kDa protein to hydrolyse ATP was investigated. The specific activity in terms of phosphate release after ATP hydrolysis was determined as 1.84 $\mu\text{mol}/\text{min}$ per μmol of 30 kDa protein.

DHFR can interact with the 30 kDa protein

For this experiment, unfolded DHFR was permitted to form a complex with the 30 kDa protein in the complete absence of substrate (Figure 5A). Subsequently the reaction mixtures were incubated for various times in the presence or absence of dihydrofolate before being assayed for DHFR activity. In the absence of dihydrofolate (Figure 5A, trace a), the 30 kDa protein–DHFR complex remained stable even after 4 h at room temperature. In contrast, a significant dissociation of complex occurred during preincubation periods with dihydrofolate (Figure 5A, traces b and c). This dissociation occurred slowly and was complete by 4 h. These results show that the 30 kDa protein DHFR complex is stable and that either substrate (NADPH or

Figure 4 Gel filtration of the 30 kDa protein

The purified 30 kDa liver protein was analysed by HPLC on a column of TSK gel G3000SW. The retention time of the protein is shown. Inset: calibration curve with standard proteins. 1, ovalbumin (43 kDa); 2, BSA (67 kDa); 3, aldolase (158 kDa); 4, catalase (232 kDa); 5, ferritin (440 kDa). The arrow indicates the 30 kDa protein.

dihydrofolate) can alter the observed equilibrium between bound and free DHFR. As shown in Figure 5(B), when unfolded DHFR was diluted 1:100 into a cuvette containing its natural substrates, enzymic activity was observed immediately (Figure 5B, trace f). Thus the oxidation of NADPH, in the presence of dihydrofolate, reaches a maximal rate comparable to that exhibited by the native enzyme (Figure 5B, trace e). The spontaneous refolding of DHFR occurs very rapidly under the conditions employed. The 30 kDa protein retarded the regaining of DHFR activity (Figure 5B, trace c), and a nearly complete arrest of refolding was observed at a high molar ratio (Figure 5B, trace b). Under these conditions, the enzymic activity of native DHFR was not significantly influenced by the presence of even the largest concentration of the 30 kDa protein tested (Figure 5B, trace d). We investigated whether the interaction with DHFR is ATP-dependent or not (Figure 5C). Dissociation of the 30 kDa protein–DHFR complex was found in the presence of ATP (Figure 5C, trace b), but not in its absence (Figure 5C, trace a).

Resistance of the 30 kDa protein to protease

Pig 30 kDa protein was mildly digested with Tos-Phe-CH₂Cl-treated trypsin by the method described in the Materials and methods section (Figure 6). In the absence of ATP or divalent cations the protein was mildly digested by Tos-Phe-CH₂Cl-treated trypsin under the experimental conditions used (protein-to-enzyme ratio 500:1). The 30 kDa protein was partly digested; the new protein band showed a molecular mass of 23 kDa on SDS/PAGE (Figure 6, lane 2 from the left). In the presence of 5 mM ATP the digestion was inhibited and a faint band of 23 kDa was observed. The divalent cations Mg²⁺ or Ca²⁺ affected the protease digestion of the protein. These divalent cations at 5 mM protected the enzyme from digestion to some extent, as did ATP. The effect of ATP on digestion was enhanced in the presence of Mg²⁺ or Ca²⁺. The 30 kDa protein was shown to be intact under these conditions. The same phenomena were observed on digestion with another protease (V₈ protease) (results not shown). ATP causes protease resistance of the 30 kDa protein in the presence of different proteases.

Figure 5 Interaction between DHFR and the 30 kDa liver protein

(A) Denatured DHFR was rapidly diluted to a final concentration of $0.2 \mu\text{M}$ in a solution containing 50 mM Tris/HCl, pH 7.4, 5 mM MgCl_2 , 3.3 mM KCl and 10 mM dithiothreitol, with (traces a–c) or without (trace d) the 30 kDa protein ($1.0 \mu\text{M}$). Some reactions (traces b and c) were then provided with dihydrofolate (0.1 mM). After a 2 h (trace b) or 4 h (traces a, c and d) incubation period all reactions were supplemented with dihydrofolate and NADPH, each to a final concentration of 0.1 mM. Reaction mixtures were then transferred to cuvettes and A_{340} was monitored ($t = 0$). (B) Reactions were conducted in a solution containing 50 mM Tris/HCl, pH 7.4, 5 mM MgCl_2 , 3.3 mM KCl, 10 mM dithiothreitol, 100 μM dihydrofolate and 75 μM NADPH. Some reactions also contained the 30 kDa protein at 0.4 μM (trace c) or 1.5 μM (traces b and d). The reaction shown in trace a also contained methotrexate (0.5 μM). At $t = -10$ s, reactions were inhibited by the rapid addition of 5 μl of native DHFR (traces a, d and e) or unfolded DHFR (traces b, c and f) to a final concentration of 0.2 μM . After vigorous mixing, reactions were transferred to cuvettes and A_{340} was monitored ($t = 0$). (C) Reactions were conducted under the same conditions as described for (B). Some reactions also contained the 30 kDa protein at 1.0 μM (traces a and b) or 0 μM (trace c). At $t = -10$ s, reactions were initiated by the rapid addition of unfolded DHFR to a final concentration of 0.2 μM . Reactions were transferred to cuvettes and A_{340} was monitored ($t = 0$); 1 min later (upward arrow) the following additions were made: trace a, Tris/HCl, pH 7.4, to a final concentration of 2.5 mM; trace b, ATP to a final concentration of 2.5 mM; trace c, no addition.

Figure 6 Protease resistance of the 30 kDa protein

Pig liver 30 kDa protein was mildly digested with Tos-Phe- CH_2Cl -treated trypsin (protein-to-enzyme ratio 500:1) in the presence (+) or absence (–) of 5 mM MgCl_2 , 5 mM CaCl_2 and 5 mM ATP for 30 min at 37 °C. After digestion, the samples were subjected to SDS/PAGE [12% (w/v) gel]. The gels were stained with Coomassie Brilliant Blue.

Figure 7 Effect of ATP on the CD spectrum of the 30 kDa protein

The CD spectrum of the 30 kDa protein was measured in the absence (solid line) or presence of 0.5 mM ATP and 5 mM MgCl_2 (dot-dashed line) at 24 °C as described in the Materials and methods section.

Effect of ATP on the CD spectrum of the 30 kDa protein

The CD spectrum of the 30 kDa protein showed an ellipticity maximum below 195 nm and two negative bands at 210 and 220 nm (Figure 7); the addition of MgCl_2 and ATP at final concentrations of 5 and 0.5 mM respectively gave rise to a single negative band at 219 nm. Control experiments showed no significant change in the CD spectrum of the 30 kDa protein if MgCl_2 was added alone. A conformational change of the 30 kDa protein was induced and the β -structure of the protein was increased after the addition of ATP, whereas the α -helical content was slightly decreased. Thus ATP induced a positive contribution

to the overall ellipticity of the 30 kDa protein in the region 195–220 nm.

DISCUSSION

We recently purified and characterized a mammalian HSP60 (chaperonin homologue) using ATP-Sepharose column chromatography [14]. The protein showed chaperone activity and was located in both the cytoplasm and mitochondria. At the same time we identified and characterized a 30 kDa ATP-binding protein from pig liver cytosol during the purification of HSP60. The partial amino acid sequences of the purified 30 kDa protein (95 residues, about one-third of the protein) had no similarity to any reported proteins in the NBRF-PIR and SWISS-PROT data

banks. These results indicate that the protein is novel. The protein also showed an affinity for Green A that was the same as for ATP. The Green A column was effective in the purification of the 30 kDa protein. The molecular mass of the protein was calculated to be 30 kDa, both by SDS/PAGE and gel filtration, indicating that the protein is a monomer. The protein was also purified from pig kidney cytosol by the same method (results not shown).

It has been shown that the ATP-binding consensus sequence is divided into two short elements termed type A and B [21,22]. The type A sequence, which denotes the putative triphosphate-binding sequence, is A/GXXXXGKT/SXXXXXXI/V. In contrast, the type B sequence, which denotes the adenine-binding sequence, is H/R/KX₍₅₋₇₎θXθθD/E, where θ stands for a hydrophobic residue. It has also been reported that the triphosphate-binding sequence and adenine-binding sequence of rat HSP70 are AEAYLGKTVTNAV (residues 131–144) and KNQV-AMNPTNTVFD (residues 56–69)/KKVGAERNVLIFD (residues 187–199)/KSENVQDLLLD (residues 384–395) respectively [23]. On the basis of the consensus sequence, the triphosphate-binding sequence of the 30 kDa protein might be **GHLGK** (lysyl endopeptidase digest 30). We could not confirm the adenine-binding sequence in the lysyl endopeptidase digests of the 30 kDa protein. Several important questions regarding this protein remain unanswered. Foremost is the question of its physiological significance. We evaluated whether or not the 30 kDa protein can form a complex with unfolded DHFR. In preliminary experiments, CD measurements indicated that DHFR is essentially devoid of secondary structure when incubated at 24 °C in 5 M guanidine hydrochloride. This unfolded DHFR was used as a substrate for refolding experiments. The interesting finding was that unfolded DHFR can form a stable complex with the 30 kDa protein in the same way as those with HSP60 [14] and GroEL [19]. The 30 kDa protein suppresses the spontaneous refolding of chemically denatured DHFR. NADPH and dihydrofolate also seem to liberate DHFR from its complex with the 30 kDa protein. This process is, slow, however, requiring several hours. DHFR could be dissociated from the 30 kDa protein complex by the addition of ATP. These results suggest that the 30 kDa protein possesses a chaperone activity. The protein might interact with early intermediates in the protein-folding pathway and mediate the acquisition of the native structure of newly synthesized proteins by releasing the substrate in an ATP-dependent process in the manner of a chaperonin (GroEL) [24,25].

The protein also showed resistance to protease in an ATP-dependent manner. Under the experimental conditions used, the protein was mildly digested by V₈ protease or Tos-Phe-CH₂Cl-treated trypsin. ATP protected the protein against these proteases. The physiological ATP conditions protected the protein from protease digestion. Divalent cations enhanced the protective effect of ATP. The reason why ATP bestows protease resistance on the protein is obscure. The CD spectrum of the

30 kDa protein is similar to those obtained from other members of the α/β structural class. However, the far-UV CD spectrum of the 30 kDa protein was significantly different from that measured in solution in the presence or absence of ATP. The overall change in CD spectrum induced by ATP might reflect an increase in β-structures and a decrease in α-helical structures of the 30 kDa protein. The secondary structure of the protein might be changed by the addition of ATP, resulting in its masking the digestion sites of the protein.

The results presented here indicate that the 30 kDa pig liver protein possesses an affinity for ATP, chaperone activity, and a protective effect by ATP against some proteases. It is still not known whether the protein functions *in vivo* as a catalyst of protein folding or merely acts as a 'molecular chaperone'. We are searching for the true physiological functions of the protein.

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