MATERIALS AND METHODS

Purification of chaperonins:

A. Bacterial strain and growth conditions:

Chromatium vinosum was obtained from the American Type Culture Collection (ATCC) in Rockville Md. USA. The bacterium was grown heterotrophically as described by Olson et al. (1973). Precultured cells were used to inoculate 20 L carboys, and were allowed to grow for five days at 30°C-35°C with continuous illumination. The bacterial cells were harvested by centrifugation at 17000 x g in an angular rotor (Sorvall-GSA) for 10 min at 4°C. The cells were then resuspended and washed with extraction buffer [50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1.0 mM MgCl₂, 0.1 mM ethylenediamine tetraacetate (EDTA), 1 mM 2-mercaptoethanol, 50 mM NaHCO₃, pH 7.3]. After washing, the cells were collected by centrifugation at 17000 x g for 10 min at 4°C in a GSA rotor. The cells were then weighed and stored at -35°C.

B. Purification Steps:

All the steps were carried out at 4°C, unless otherwise indicated. Frozen bacterial cells (approximately 60 g) were resuspended in 150 mL of extraction buffer containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 10 μg each of DNAase and RNAase. The cells were then disrupted by sonic treatment using a Vibra cell unit (VC 250 by Sonics and Materials Inc.) with five 1-min
treatment periods at full power in an ice bath. Cellular debris was removed by centrifugation at 18000 x g for 10 min in a Sorvall SS-34 rotor. Then the supernatant was collected and diluted (1:1 v/v) with extraction buffer and enough polyethylene glycol (PEG)-8000 was added from a stock solution (60% w/v) to obtain a final concentration of 10% PEG. The solution was stirred for 50 min and then subjected to centrifugation at 18000 x g for 30 min (in a Sorvall SS-34 rotor). Sufficient 1 M MgCl$_2$ was added to the supernatant to obtain a final concentration of 50 mM and the solution was stirred for 30 min. Then, the suspension was subjected to centrifugation at 18000 x g for 20 min (in a Sorvall SS-34 rotor). The pellets were collected and resuspended in 12 mL of extraction buffer. Two mL of the protein solution was applied to each of six 38 mL linear sucrose density gradients (0.2 M to 0.8 M) using Beckman quick-seal centrifuge tubes. The gradients were then subjected to centrifugation at 243,000 x g for 140 min in a Beckman VAC 50 rotor. Fractions of 1.5 mL were collected using a fraction collector (Retriever II, by ISCO) in a cold room. The absorbance was measured at 280 nm in a Beckman DU-7 Spectrophotometer. In order to examine the homogeneity of the preparation, the collected fractions were analyzed by 15% SDS-PAGE. A second sucrose gradient was conducted to further purify the proteins of interest.
**SDS - Polyacrylamide Gel Electrophoresis:**

Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate (SDS) was performed in a gel slab polymerized from 15% Bio-Rad ultrapure-grade acrylamide (Laemmli, 1970). Samples containing 100 μg of protein were subjected to electrophoresis. The electrophoresis was initially carried out at 100 volts until the tracking dye [0.01% (w/v) bromophenol blue] reached the resolving gel and then, the voltage was changed to 250 volts until the tracking dye reached the bottom of the gel. The gel was stained in a mixture of water/methanol/acetic acid (5:5:1 v/v/v) containing 0.1% (w/v) Coomassie Brilliant Blue, overnight, and then it was destained with the mixture of water/methanol/acetic acid.

Subunit molecular weights were determined by comparing Rf values with those of low-molecular-weight standards (Bio-Rad, Laboratories, Richmond, CA). The protein molecular weights are: B-galactosidase (116,250), phosphorylase B (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400) and aprotinin (6,100).

**Development of polyclonal antibodies:**

Polyclonal antibodies to the cpn10 and cpn60 were developed in New Zealand rabbits by the following method. Briefly, protein bands in gels after SDS-PAGE corresponding to cpn10 or cpn60 were excised and macerated in
the presence of 0.5 mL complete Freund's adjuvant. The resulting emulsion was injected subcutaneously in the neck region with the first booster given after 4 weeks. Additional booster injections, in incomplete Freund's adjuvant were administered, as necessary, every two weeks and antibody titers were checked after blood withdrawal. The blood was obtained by bleeding from the ear or by cardiac puncture (Vierling et al., 1989). Cellular material was removed from the serum by centrifugation at 298 x g for 5 min at room temperature. Specificity of the antisera was confirmed by Western immunoblotting analysis.

Preparation of a rat liver and pancreas cell-free extract:

A rat liver and pancreas cell-free extract was prepared by homogenizing 1 g of fresh hepatic and pancreatic tissues in 2 mL of a lysis buffer [20 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, 1mM PMSF and 2% (w/v) soybean trypsin inhibitor, pH 8.0] at 4°C utilizing a Ten Broeck tissue grinder. The resulting homogenate was diluted (1:3 in lysis buffer) and subjected to centrifugation at 48 x g for 15 min to remove cell debris. The supernatant was used for Western immunoblotting analysis. Protein concentration was determined by Bradford's method (Bradford, 1976). Polyacrylamide gel electrophoresis was conducted in slab gels in the presence of sodium dodecyl sulfate as described previously. All the reagents were obtained from Sigma Chemical Co. (St. Louis, MO., USA).
Western Immunoblotting:

Polyclonal antibodies against the cpn10 and cpn60 from *C. vinosum* developed in New Zealand rabbits were used. The monoclonal antibody against the hsp70 protein was purchased from Sigma (Sigma Co., St. Louis, MO). Blotting of electrophoresed proteins onto nitrocellulose membrane and, immunodetection of cpn10, cpn60 and hsp70 were performed as described by Towbin (Towbin et al., 1979). In short, after electrophoresis, the gels were placed adjacent to a Bio-Rad sheet of nitrocellulose membrane [pretreated with Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/v) methanol, 10% (w/v) SDS, pH 9.2)] and electroblotted for 45 min in a Transblot SD semidry electrophoretic transfer cell (Bio-Rad, Richmond, CA) at 23 volts. After transfer, the membrane was incubated with blocking buffer [5% (w/v) non-fat dry milk in saline buffer (10.5 mM Tris-HCl and 257 mM NaCl, pH 7.5) (TBS)] for 2 hr at 37°C. Then, the membrane was washed with distilled water. Blocking buffer containing primary antibodies against cpn10, cpn60 and hsp70 were used at 1:200, 1:150 and 1:500 dilution, respectively, in TBS buffer overnight. The nitrocellulose membrane was washed three times with Tween 20 [0.05% (w/v)] in TBS buffer at 5 min intervals and then, three times with distilled water. The membrane was then incubated for 2.5 hr at 25°C in blocking buffer containing alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Co., St Louis, MO, USA) at 1:1000 dilution (secondary antibody). For
the detection of hsp70, an additional step included an incubation with rabbit anti-mouse IgG serum (Miles laboratories, Inc., Illinois, USA) at 1:1000 dilution before the addition of alkaline phosphatase-conjugated goat anti-rabbit IgG. After incubation the membrane was washed as described before. The specific binding of the primary antibodies was revealed in an alkaline buffer (100 mM Tris-HCl, 100 mM MgCl₂, pH 9.5) containing the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP, Boehringer kit, Boehringer-Mannheim, Laval, Canada). The reactions were quenched by rinsing the blots several times with distilled water.

Crystal induction:

Adult male Sprague-Dawley rats 200 g in weight, received a single intraperitoneal injection of 50 mg/100 g body weight of DL-p-chlorophenylalanine methyl ester (CPME) dissolved in saline to induce the formation of intracisternal crystals as previously reported (Arias and Bendayan, 1993; Forssman and Bieger, 1973), whereas control animals received saline alone.

Immunohistochemistry:

For light microscopy, rat hepatic and pancreatic tissue was fixed with Bouin's solution for 24 hr at room temperature and embedded in paraffin according to standard procedures. Tissue sections (5 μm thick) were deparaffinized in xylene, rehydrated in graded ethanol solutions, washed in 0.01 M phosphate-
buffered saline (PBS), pH 7.4, and incubated overnight at 4°C with either the anti-cpn10, the anti-cpn60 or the anti-hsp70 antibodies diluted 1:10 in PBS. The tissue sections were then rinsed with PBS and incubated for 60 min at room temperature with a fluorescein labeled anti-rabbit IgG antibody (1:50 dilution) for cpn10 and cpn60 or, fluorescein labeled anti-mouse IgG (1:50 dilution) for hsp70. After final washings with PBS and distilled water, sections were counterstained using 0.1% (w/v) Evans blue for 5 min, and mounted. The sections were examined with a Leitz ortoplan microscope using a Ploemopak illuminator.

Control experiments included: a) preadsorption of the primary antibody with an excess of the corresponding antigen, purified cpn10 and cpn60 proteins from C. vinosum or hsp70 protein (Sigma, Co., St. Louis, MO.) and b) omission of the primary antibodies.

Immunocytochemistry:

For electron microscopy, C. vinosum cells and small fragments of hepatic and pancreatic tissue from normal and CPME-treated animals were fixed by immersion with 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 2 hr at 4°C, dehydrated in a series of graded methanol solutions and embedded in Lowicryl K4M at -20°C as described previously (Bendayan, 1984). The protein A-gold or the protein AG-gold immunocytochemical techniques (Bendayan, 1984; Ghitescu et al., 1991) were applied on thin sections for the
localization of the cpn10, cpn60 and hsp70 antigenic sites. In CPME-treated animals amylase was used to reveal antigenic sites. Briefly, thin sections mounted on nickel grids were incubated by floating the grids successively on a drop of PBS for 5 min and 1% (w/v) ovalbumin (Sigma Chemical, St. Louis, MO., USA) in PBS for 60 min at room temperature. They were then transferred to a drop of the primary diluted antibody (anti-cpn10, anti-cpn60 or anti-hsp70 at 1:10 dilution) and incubated at 4°C overnight. The tissue sections were then rinsed with PBS, transferred to 1% (w/v) ovalbumin for 60 min, and then incubated for 30 min on a drop of the protein A-gold complex for the cpn10 and cpn60, or a drop of the protein AG-gold complex for the hsp70. The grids were then thoroughly washed with PBS, rinsed with distilled water, dried and stained with uranyl acetate and lead citrate, before examination with a Philips 410 electron microscope. Both the protein A-gold and protein AG-gold complexes were prepared with 10 nm gold particles as described previously (Ghitescu and Bendayan, 1990; Ghitescu et al., 1991). In the pancreas, acid phosphatase was localized using cytidine-5'-monophosphate as substrate (Novikoff, 1963), followed by Lowicryl embedding.

In order to assess the presence of chaperones in pancreatic tissues from other species, thin sections of human, mouse and guinea pig pancreas fixed in 1% (w/v) glutaraldehyde and embedded in Lowicryl K4M were immunolabeled as described above.
For fracture-labeled cytochemistry, rat liver tissue was fixed by immersion in 1% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 hr at 4°C. It was then washed in 0.1 M cacodylate buffer, trimmed into 1-mm cubes and embedded in 30% (w/v) bovine serum albumin (BSA, Calbiochem; La Jolla, CA, USA) which was cross-linked with 1% (v/v) glutaraldehyde to form a hardened gel (Kan and Pinto da Silva, 1986; Pinto da Silva et al., 1981). Frozen liver tissue embedded in BSA gels was fractured and further processed according to the method previously described (Kan and Pinto da Silva, 1986; Pinto da Silva, 1981). Immunolabeling was carried out after freeze-fracture and thawing by incubation with the anti-cpn10 and anti-cpn60 antibodies overnight (1:10 dilutions) at 4°C, followed by labeling with a protein A-gold complex for 1 hr at room temperature. The gels were washed with PBS and further fixed with 1% (v/v) glutaraldehyde in PBS before drying by the Pelldri II sublimation method (Kan, 1990). Freeze-fracture replicas were prepared according to a procedure previously described (Kan and Bendayan, 1989) and examined with a Philips 410 electron microscope.

Control experiments for assessing the specificity of the immunolabelings included: a) preadsorption of each antibody with its corresponding antigen prior to the labeling, and b) incubation with the protein A-gold complex alone, and c) incubation with the antibody followed by native protein A and the protein A-gold complex (Bendayan, 1984).
Quantitative evaluation:

The labeling intensities for cpn10 and cpn60 obtained over the mitochondria and peroxisomes on thin sections from rat liver tissue as well as the cpn10, cpn60 and hsp70 over the different cellular compartments from rat pancreatic tissue were defined as the number of gold particles per μm². They were obtained by direct planimetry and counting of gold particles (Bendayan, 1984). Micrographs of 15 different cells from each labeling experiment were recorded at 26000x final magnification. In addition, the exact location of the gold labeling for both cpn10 and cpn60 on bacteria as well as on mitochondria and peroxisomes was also assessed. The shortest distance between each individual gold particle and the bacterial envelope or mitochondrial cristae membrane or the limiting membrane of peroxisomes was measured and their distribution determined. Fifteen electron micrographs at 77000x final magnification were evaluated. On the other hand, quantitative evaluations of the labelings present over the RER, Golgi area, condensing vacuoles, zymogen granules, mitochondria and RER intracisternal crystals were performed for both chaperonins and the hsp70 in rat pancreatic tissue. A Videoplan 2 image processing system (Carl Zeiss Inc, Toronto, Ontario, Canada) was used for the analysis. Additionally, in the RER intracisternal crystal studies, statistical comparisons of the densities of labeling were performed using an ANOVA and Student t tests for the control and CPME-treated animals.