Characterization of a Novel Complex from Halophilic Archaebacteria, Which Displays Chaperone-like Activities in Vitro*

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We isolated a protein, P45, from the extreme halophilic archaean Haloarcula marismortui, which displays molecular chaperone activities in vitro. P45 is a weak ATPase that assembles into a large ring-shaped oligomeric complex comprising about 10 subunits. The protein shows no significant homology to any known protein. P45 forms complexes with halophilic malate dehydrogenase during its salt-dependent denaturation/renaturation and decreases the rate of deactivation of the enzyme in an ATP-dependent manner. Compared with other halophilic proteins, the P45 complex appears to be much less dependent on salt for its various activities or stability. In vivo experiments showed that P45 accumulates when cells are exposed to a low salt environment. We suggest, therefore, that P45 could protect halophilic proteins against denaturation under conditions of cellular hyposaline stress.

The survival of a cell is critically dependent on its ability to adapt rapidly to changes in the natural environment. Halophilic archaebacteria that live in environments where salt has been concentrated by evaporation have to face an unusual type of stress. These organisms balance the extremely high external salt concentration by accumulating multinuclear KCl concentration in their cytosol (1). Their proteins are themselves halophilic and function in conditions where “normal” proteins would denature or aggregate because of low water activity and strong salting out effects (for review, see Ref. 2). The stabilization and solvation of halophilic proteins are related to the cooperative interaction of acidic surface residues with hydrated solvent ions (3). Halophilic proteins are salt-binding proteins that unfold when the salt concentration decreases. It is commonly accepted that in the crowded cytosolic environment of all types of cells, the accumulation of exposed hydrophobic surfaces causes irreversible aggregation of misfolded proteins which ultimately leads to cell death (4, 5). Therefore, a decrease in salt concentration could represent a major stressor for halophilic Archaebacteria that are exposed to hyposaline shock after rain or flooding. Very little is known about the antistress mechanisms that enable these cells to avoid the accumulation of misfolded proteins in the cytosol during low salt shock. Cells usually respond to various stressors with the synthesis of a distinct set of proteins known as heat-shock proteins (Hsps), which are ubiquitous in organisms ranging from Escherichia coli to humans (6, 7). Most of the stress proteins assist in protein folding as molecular chaperones (8), which share the general property of interacting with many non-native proteins and influencing the conformational state of the bound proteins in an ATP-dependent manner (9). It is commonly accepted that these properties are also involved in unstressed cells in facilitating protein synthesis, folding, and assembly processes.

Among the chaperones, the Clp/Hsp100 family and Hsp60 play a key role as antistress factors by preventing the aggregation of a large spectrum of improperly folded and damaged proteins (10–12). Hsp100 self-assembles in oligomeric rings whereas Hsp60s, also called chaperonins, are rod-shaped particles made up of double stacked rings (13, 14). These large oligomeric complexes create hydrophobic cellular subcompartments where misfolded proteins can be trapped. The small heat-shock proteins (sHsps) also consist of large oligomeric complexes that trap misfolded proteins (15). However, their role under stress conditions is confined to creation of a reservoir of non-native refoldable proteins. These can eventually be refolded to the native state in cooperation with true chaperones in an ATP-dependent reaction (16, 17). In addition to their antiaggregation effect, Hsp100 and chaperonin complexes have been shown in vivo and in vitro to function in stress conditions by slowing down the protein denaturation process in an ATP-dependent manner (18, 19). Interestingly, there appears to be an interplay between protein folding and hydrolysis as illustrated by the fact that members of the Hsp100 chaperone family can also associate with the proteasome or its bacterial ClpP counterpart and thus regulate proteolysis through an ATP-dependent unfoldase activity (20).

The study of stress proteins in Archaea is much less advanced than in the other two domains (for review, see Ref. 21). Archaea are prokaryotes that, based on phylogenetic studies and their biochemical properties, can be grouped in a third kingdom of life distinct from those of the Bacteria and the Eukarya (22, 23). Chaperones of the Hsp100 class occur in Archaea as was shown by the characterization of an homolog of the eukaryotic 26 S proteasome-activating complex in Methanococcus jannaschii (24, 25). A chaperonin system exists in all archaeal genomes investigated so far (21). It has been studied mainly in the thermophilic Archaea where it has been named thermosome (26, 27) and found to be related to the eukaryotic chaperonin system TCP-1 (28). The thermosome can

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1 The abbreviations used are: Hsp(s), heat-shock protein(s); sHsp, small heat-shock protein; HmMalDH, halophilic malate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.
function autonomously, in contrast to the bacterial chaperonin,
GroEL, which requires the binding of a cochaperonin complex,
GroES, for the release of the protein substrate (29). Little is
known about the role of these macromolecular machines in the
specific salt stress response of halophilic Archaea. The genes
coding the thermosome subunits from the halophile
Haloferax volcanii (ect1 and ect2) were found to be induced both
by heat and hyposaline shock (30). This suggests that the
conventional heat-shock system could also be adapted to func-
tion during low salt stress in extreme halophiles. However, a
number of other potential stress response factors have been
detected on two-dimensional gels, suggesting the existence of a
defense system specific for salt stress (31). In a attempt to
characterize such a system, we describe here the purification of
a new ring-shaped ATPase complex from the extreme halophi-
lic Archaeon Haloarcula marismortui. To characterize its
function, we used the malate dehydrogenase enzyme
(HmMalDH) from the same organism as a natural substrate for
in vitro chaperone assays. HmMalDH can be unfolded and
refolded in vitro by a simple modification of the solvent salt
concentration. We were able to demonstrate that the complex,
which is not homologous to any known chaperone, displays
polypeptide binding capability and hampers the low salt dena-
turation of the halophilic enzyme in an ATP-dependent man-
er. In vivo experiments showed that the P45 protein, which
forms the complex, is induced when halophilic cells are exposed
to a low salt stress. P45 has, therefore, the characteristics of a
molecular chaperone involved in stress response.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Medium—**H. marismortui cells were kindly
provided by A. Oren (University of Jerusalem). Cells were cultivated in
2-liter flasks at 37 °C with gentle agitation in a growth medium con-
taining 3.5 mM NaCl (32). Cells were harvested when the A600 nm
reached 2 by centrifugation for 1 h at 12,000 g and stored at −80 °C until use.

**Experimental Procedures**

**Haloarcula marismortui** strain was from the laboratory of D. Oester-
heft (Max Planck Institute, Munich). Cells were cultivated as described
by Oesterhardt and Stoeckenius (33).

**Proteins—**The HmMalDH gene from H. marismortui was expressed in
E. coli, and the protein was purified and renatured as described previously (34).
The E. coli GroEL and GroES recombinant proteins were purchased from Roche Molecular Biochemicals. Bovine
serum albumin was from Sigma.

**P45 Protein Purification and Sequencing**—Cell pellets (correspond-
ing to about 30 liters of culture) were thawed and resuspended in 1
volume of buffer A (50 mM Tris/HCl, 2.2 mM (NH4)2SO4, 40 mM MgAc2, pH
7.6) containing 20 mg of DNase I grade II from Roche. After a 30-min
incubation at room temperature, the cell lysate was homogenized by
ultrasonication (6 cycles of 10 s) and centrifuged for2 h at 160,000 g.
The supernatant was centrifuged for 2 h at 160,000 g. The S160 super-
natant was dialyzed for 48 h at 4 °C versus buffer A and then loaded on
a Superose 4B (Amersham Pharmacia Biotech) 5.5 × 30-cm column
equilibrated in buffer A. The protein was eluted with a decreasing
linear (NH4)2SO4 gradient (2.2–0.4 M in a total of 950 ml of buffer A).
The P45 protein was detected by immunoblot in the fractions at −0.9 M
(NH4)2SO4. Solid ammonium sulfate was added to increase the concen-
tration of the pooled fractions to 1.9 M. The sample was applied to a
deAE-2 cellulosic column (2.6 × 25 cm) equilibrated in 50 mM Tris, 2
mM (NH4)2SO4, pH 7.6. Bound proteins were eluted using a decreasing
(NH4)2SO4 gradient (1.9–0.5 M) and an increasing NaCl gradient (0–2 M)
over 500 ml. The fractions containing P45, obtained at 0.8 M NaCl, 1.2 M
(NH4)2SO4, were pooled and dialyzed against 20 mM sodium phosphate buffer containing 4 M NaCl. The protein sample was applied to a
column (1.5 × 10 cm) of hydroxyapatite (Bio-Rad) preequilibrated with
the dialysis buffer. The protein was eluted at 18 ml/h using a
200-ml linear phosphate gradient from 20 to 300 mM phosphate in 4 M
NaCl. The fractions containing P45 were dialyzed against 20
mM Tris-HCl, pH 7.6. The protein mixture was loaded on a Mono Q HR
5 × 5 column (Amersham Pharmacia Biotech) and eluted with a 20-ml
linear gradient from 0 to 2 M KCl in 20 mM Tris, pH 7.6. P45 was eluted at
1.1 M KCl. The fraction was dialyzed against 3 M KCl, 20 mM Tris, pH
7.6, and contained native P45 protein complex purified homogeneously
as judged by mass spectrometry and analytical centrifugation analysis.

Protein concentrations were determined by the method of Bradford (35)
using bovine serum albumin as the standard. SDS-PAGE was
performed according to Laemmli (36). The protein was immunodetected in
the fractions as described previously by Franzetti et al. (37) using
antibodies raised against a synthetic peptide deduced from the N-
terminal peptide and four others were sequenced using a protein sequencer.

**Analytical Ultracentrifugation—** Sedimentation velocity experiments
were performed using a Beckman XL-A analytical ultracentrifuge and
an AN-60 rotor (Beckman Instruments). 300 μl of diluted protein at
about 0.15 mg/ml (A280 nm = 0.07) in 3 M KCl, 20 mM Tris-HCl, pH 7.6,
was loaded into two-channel 1.2-cm path cells and centrifuged with a
rotor speed of 42,000 rpm at 20 °C. Scans were recorded at 227 nm
using 0.003-cm radial spacing.

Using Sednterp software (version 1.01; developed by D. B. Haynes, T.
Laue, and J. Philo) we estimated the solvent density ρ to be 1.132 g/ml,
the solvent viscosity η to be 1.019 mPas. Direct boundary modeling of
the sedimentation profiles by Lamm equation solutions was applied
using the program Sedfit (39) considering two noninteracting species
and taking advantage of a procedure of algebraic systematic noise
decomposition (40). These programs can be downloaded from www.b-
bri.org/RASM/Brasnsh.html. In the framework of the model of analysis
both the sedimentation and diffusion coefficients s and D are obtained.
The diffusion coefficient is related to fβ, the frictional ratio, expressing
the asymmetry of the macromolecule. The Svedberg equation
obtaining from s and D the buoyancy molar mass M, from which
the molar mass M can be derived.

Because the solvent is composed of two components, an atomic
partial specific volume ϕ is used instead of the usual partial specific
volume V. We consider ϕ = 0.72 ml/g, which is the mean value obtained
in 3 M KCl for halophilic polypeptide elongation factor Tu (41) and
HmMalDH (3). V and ϕ are close because the composition of the
solvation shell is closed to that of the bulk solvent for these halophilic
proteins. The corrected s20,w was obtained using p20,w = ρ20,w and
η20,w, the density and viscosity of water at 20 °C.

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M_w = M \cdot (1 - \phi f) = RTsD
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measured as described (43). The activity measurements were carried out at 40 °C in 50 mM Tris-HCl, 2 mM KCl, pH 7.6.

Binding Assays of HmMalDH on P45 or GroEL—The denaturation or renaturation mixtures (final volume, 50 μl) were prepared and incubated for 1 h as described above. Size exclusion chromatography with samples containing P45 w/o GroEL were run on a SW 6000 column (0.78 × 60 cm, TosohHaas) mounted on a HPLC chain with a flow rate of 0.9 ml/min. The samples containing GroEL were analyzed with a Superose 6 (HR 10 × 30) column (Amersham Pharmacia Biotech) with a flow rate of 0.5 ml/min. Running buffers were the same as the denaturation and renaturation buffers. The proteins were precipitated from the fractions with 12% trichloroacetic acid using tRNA as the carrier. Precipitates were washed with cold acetone and subjected to SDS-PAGE followed by Western blotting with antibodies against HmMalDH. The immunoreactivity of the protein was detected by using the antibodies against GroEL or P45.

In Vivo P45 Expression—To investigate the accumulation of the P45 protein under stress conditions, H. marismortui cultures (A_{660} = 0.7–0.8) growing in complex hypersaline medium containing 3.6 M (20.8%) NaCl were subjected to reduced salt concentration. The cells were pelleted, resuspended in medium containing 1.35 M (5%) NaCl, and incubated at 37 °C with shaking. A time course was effectuated in which cell aliquots of 1 ml were removed at 1, 2, 4, and 6 h. Cells were pelleted and resuspended in a liquid nitrogen. The cells were resuspended in 0.4 ml of a buffer containing 100 mM Tris-HCl, pH 8, 0.1% Triton X-100, 5 μg/ml RNase, and 10 μg/ml DNase. The lysates were incubated for 10 min at room temperature, and cell debris were eliminated by centrifugation. Western blot analysis was performed as described by Franzetti et al. (37). Samples loaded into the gel contained equal amounts of total proteins. The P45 protein was immunodetected by using the antibodies raised against the N-terminal peptide. The blots were reprobed with polyclonal antibodies raised against HmMalDH and the catalase peroxidase enzymes from H. marismortui.

RESULTS

Purification of a 45-kDa Protein from H. marismortui and Identification of the Homologous Gene in H. salinarium—H. marismortui cells, which normally grow optimally in salt concentrations between 3 and 4 M NaCl, were progressively adapted to lower salt conditions such as 2.5 M NaCl. SDS-PAGE of total protein from these cells revealed an abundant protein with an apparent molecular mass of 55 kDa. The protein was first purified from this strain as described under “Experimental Procedures.” Polyclonal antibodies were raised against the N-terminal peptide of the protein, allowing its detection in the chromatography fractions. Because the adapted strain that was growing in low salt conditions could not be stored or maintained for a long period, the protein was purified subsequently from wild type H. marismortui cells that were grown under normal hypersaline conditions. The N-terminal sequence of the protein purified from the wild-type cells was found to be identical to the one obtained from the mutant strain.

A stained SDS-PAGE showing the different purification steps is presented in Fig. 1. All operations were performed in hypersaline conditions to maintain the solubility and the stability of the halophilic proteins before the last step of the purification where the proteins were denatured by low salt conditions before being resolved by ion exchange chromatography. This last step was necessary to obtain a high purification yield, and the resulting P45 protein shows the same oligomeric properties and biochemical activities (from electron microscopy and chaperonin assays) as those obtained when avoiding the low salt conditions, by using an alternative purification protocol in which gel filtration chromatography was used in the last step. We routinely obtained about 0.4 mg of pure protein from 60 g of cell paste.

The protein purified from H. marismortui cells was partially sequenced. This allowed the unambiguous identification of the H. salinarium homolog gene by Prof. D. Oesterhelt, who kindly agreed to screen the unpublished sequence H. salinarium genome obtained by his group. Fig. 2 shows that the sequences obtained had strong homologies with an open reading frame (AAG18988.1) coding for a protein of 45.025 kDa in the sequence extreme halophile H. salinarium genome (44). The discrepancy between the molecular mass deduced from the protein sequence and the one that we estimated on a denaturing gel is attributed to the strong excess of negatively charged acidic amino acids (the calculated P_{i} is 4.2), which affects the mobility of the protein on SDS-polyacrylamide gels, as has frequently been observed for halophilic proteins. Because no homologous sequence was found in the complete genomes of other Archaea, we concluded that the protein is, if not specific to halophilic strains, at least very well conserved in halophiles. Furthermore, the AAG18988.1 sequence had no homologies with other known proteins that were strong enough to specify the function of the protein. We concluded that we have purified a new protein, designed in this paper as P45, and we proceeded to characterize its potential functions. To compare the H. marismortui P45 protein with its H. salinarium counterpart, Western blot experiments were performed on total H. salinarium and H. marismortui extracts using the antibodies raised against the N-terminal peptide from the H. marismortui protein. The result (not shown) indicated that the H. salinarium gene was indeed transcribed and translated as a protein and that both proteins displayed similar characteristics as they migrate on denaturing gels with an identical apparent molecular mass of 55 kDa. We also purified the protein from H. salinarium with a protocol similar to the one used for H. marismortui (data not shown). Electron microscopy studies showed that the H. salinarium protein assembles in large oligomeric complexes that were similar in size and in shape to those observed with the H. marismortui protein (see Fig. 2).
H. marismortui
P45 complex. Subpanel b material). Related and modeled values. The positions of the subunits in half of the ring arrow population of ring-shaped complexes in end-on views. The dimensions of the oval shapes are 10 × 11 nm, and they might correspond to a side view of the rings. The ring shape complexes are circular, 11 nm in diameter, with a 3-nm hole in the center (Fig. 3Bc). There is always one half of the ring which appears clear, and the other is smeared out. By analyzing the clear part (see the enlargement in Fig. 3Bc) and by extrapolation to the other half of the ring, we derived that there are 2 × 5–6 monomer/rring. The total number of monomers in the complex thus appears to be 10–12.

P45 Displays an Unusual Stability in Low Salt Conditions Compared with Other Halophilic Proteins—Proteins purified from extreme halophiles have been shown to display an unusual salt-dependent solvation mechanism that leads to their denaturation in low salt conditions (below 2 M) (43). Therefore, we checked the salt-dependent stability of the P45 complex to specify its halophilic character. For this, the protein denaturation rates were studied by measuring the residual fluorescence signal at 332 nm after a 12-h incubation in different salt conditions. The data presented in Fig. 4 show that P45 requires a high salt environment to remain stable and active over long periods. Nevertheless, this halophilic feature is less pronounced than for other halophilic soluble enzymes studied previously (2); fluorescence experiments and electron microscopy studies revealed that P45 remains oligomeric and stable over 24 h in a low salt concentration such as 0.4 M, whereas most halophilic proteins, for instance HmMalDH, denature at salt concentration below 2 M KCl (3, 46).

P45 Is a Weak ATPase—Given that several classes of chaperones have an affinity for ATP (9) (47), the ability of the 45-kDa protein to hydrolyze ATP was investigated. Fig. 5 shows the variation in rates of ATP hydrolysis observed for P45 compared with those of E. coli GroEL over a wide range of KCl concentrations. The maximum specific activity in terms of P i release after ATP hydrolysis was determined as 0.75 μmol/min/μmol of P45 in a buffer containing 0.2 M KCl. This value is similar to what was found for GroEL under the same conditions. P45, therefore, has a weak ATPase activity. Interestingly, this activity was inhibited at salt concentrations above 1 M, whereas the activity of GroEL remained unaffected.

P45 Forms Complexes with Unfolded HmMalDH during Its Denaturation by Low Salt—As stress proteins, molecular chaperones interact with misfolded proteins to prevent their aggregation and to provide a protected compartment to refold properly. P45 was found to be very stable over long periods in salt conditions that constitute denaturing conditions for halophilic

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**FIG. 3. Characterization of P45 as a homo-oligomeric complex.** Panel A, sedimentation velocity experiments of purified P45 performed at 42,000 rpm. Subpanel a, profiles were modeled considering two noninteracting species with s20,w values of 6.1 and 16.4 S (85% of the material). Subpanel b, the residuals are the differences between calculated and modeled values. Panel B, electron micrographs of the purified H. marismortui P45 complex. Subpanel a, pure P45 was negatively stained with 2% uranyl acetate. The picture shows a homogeneous population of ring-shaped complexes in end-on views. Subpanel b, a possible side view of the ring, indicated by an arrow. Subpanel c, zoom view of the ring complex. The positions of the subunits in half of the ring are indicated by the white dots.

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**FIG. 4. Salt-dependent stability of P45 compared with HmMalDH.** P45 (triangles) and HmMalDH (squares) were diluted and incubated for 24 h in buffers containing various KCl concentrations. Residual intrinsic fluorescence at 332 nm obtained with an excitation at 295 nm was expressed as a function of the salt concentration.
proteins such as HmMalDH (see Fig. 4). Therefore, it was possible to study the potential role of P45 as a molecular chaperone during low salt stress, using the HmMalDH as a substrate. Nonhalophilic MalDH is known to be a good substrate for molecular chaperones such as Hsp60 (GroEL) (18). HmMalDH is a homotetramer of 32-kDa subunits which loses its activity, quaternary, and secondary structures when incubated in salt conditions below 2 M KCl (43). Fluorescence studies confirmed that a 1-h incubation in 0.4 M KCl is sufficient to deactivate and denature the enzyme completely (not shown). Conversely, about 60% of the initial activity is recovered after a 10-fold dilution in 3 M KCl as shown in Fig. 6A. This offered us the possibility of studying the role of the putative P45 chaperone complex during protein denaturation and renaturation. Because GroEL is stable and maintains its ATPase activity over a wide range of salt concentrations, it was used as a positive control in the P45 chaperoning assays.

Native HmMalDH was diluted in denaturation buffer (0.5 M KCl) with or without P45 or GroEL. In all experiments performed, the time course of inactivation was found to be accelerated slightly in the presence of either complex, but it was not affected in a control experiment where the complex was replaced by an equivalent amount of bovine serum albumin (Figs. 6A and Fig. 7 and data not shown). After dilution back to hypersaline conditions, a complete inhibition of the spontaneous reactivation was observed when P45 or GroEL was present in a 1:1 molar ratio (Fig. 6A). The percentage of inhibition was found to be proportional to the quantity of P45 added in the denaturation mixture. This suggested that, as for the chaperonins, the protection effect against reactivation was caused by the formation of complexes during the denaturation sequence between P45 and HmMalDH. To determine if complexes were formed, the protein mixtures were applied to a gel filtration column after a 1-h incubation in low salt. The chromatographic analyses were performed in the same denaturing salt conditions (0.5 M KCl). The proteins were immunodetected in the different chromatographic fractions. When the HmMalDH was incubated in the presence of P45 in a 1:1 molar ratio (i.e. one MalDH polypeptide chain for one P45 oligomeric complex, assuming a 10-subunit complex), about 50% of the protein was found to be shifted toward the fractions containing the native P45 complex, indicating the formation of a stable complex (Fig. 6B). Using larger amounts of P45 does not lead to the complication of more MalDH. In control experiments using purified GroEL instead of P45, we found that the chaperonin complex from E. coli also binds to about half of the halophilic MalDH during its denaturation by low salt. In the presence of P45 or GroEL, no regain of enzymatic activity was observed, indicating that the P45 complex binds mainly to the subpopulation of HmMalDH molecules which is competent to refold, which corresponds to the 50% that we found trapped by our complex. The results of these experiments remained unaffected by the presence of a 10-fold molar excess of bovine serum albumin in the solvent. From this we conclude that purified P45 binds specifically to unfolding intermediates.

**P45 Protects HmMalDH from Low Salt Inactivation in an ATP-dependent Manner**—The ATPase activity of P45 was found to be maximum in low salt conditions. Therefore we tested the effect of P45 on the HmMalDH inactivation in the presence of ATP. We found that P45 plus ATP slows down the rate of enzymatic inactivation significantly (Fig. 7). The molar ratio between the P45 and HmMalDH was increased up to 4:1 without obtaining a better protection against denaturation. This is consistent with the binding experiments suggesting that only 50% of the protein represents substrates for the P45 complex. The stabilization-promoting activity of GroEL during thermal denaturation has been clearly demonstrated with mitochondrial MalDH (18). This activity required the cofactor GroES and Mg\(^{2+}\)-ATP. Here we show that a similar effect could be observed with the archaeal HmMalDH during its low salt-
P45-mediated protection of HmMalDH against low salt denaturation. 1.8 μM HmMalDH was diluted in the denaturation buffer containing GroEL-ES or P45 added at an equimolar ratio with the single polypeptide chains. The ratio of GroEL to GroES was kept constant at 1:1. Where indicated, 5 mM MgATP was added to the mixture. Residual enzyme activity was measured at the indicated times.

induced denaturation. This indicates that a mesophilic chaperonin system can recognize a halophilic protein and assist its folding during low salt stress. From these experiments we concluded that P45 prevents the low salt denaturation of MalDH by an ATP-dependent mechanism similar to the GroEL/ES system.

P45 Accumulates Specifically in Low Salt-stressed H. marismortui Cells—The results obtained in vitro suggested that P45 is a stress protein susceptible to assist the folding of halophilic proteins. To strengthen this hypothesis with in vivo data, we examined the effect of salt stress on the level of P45 within cells. For this purpose, H. marismortui cells were grown in normal complex medium containing 20.8% NaCl, centrifuged, and resuspended in medium containing reduced NaCl concentration (5%). The P45 level in total protein extracts from these cells was deduced from Western analysis (Fig. 8). The results showed that the accumulation of P45 increases significantly after 1 h of exposure to stress condition and continues to rise until 4 h. The expression of two other proteins, HmMalDH and catalase peroxidase, was also studied. In both cases the levels of proteins were found to be unaffected in response to decreased salt in the external environment. From these experiments we concluded that P45 expression is specifically induced by low salt stress conditions.

P45 Interacts with HmMalDH during High Salt Renaturation—Our data indicated that P45 displayed chaperone-like activity in hyposaline stress conditions. Because P45 is also present in unstressed cells, we wanted to know whether or not P45 could also play housekeeping chaperone functions by assisting halophilic protein folding in normal hypersaline conditions. For this purpose, we examined the influence of P45 on the reaction rates of the HmMalDH. When P45 was added into the hyposaline renaturation buffer and incubated in a 1:1 molar ratio with the denatured protein, only 20% of the initial activity was recovered (Fig. 6A). If we assume that the P45 complex recognizes specifically HmMalDH during its refolding sequence and not the denatured protein, the proportion of bound protein does account for the percentage of renaturation inhibition observed (20%). This also suggests that the complex between P45 and its HmMalDH substrate is stable. The same conclusions can be drawn from the GroEL assays where a larger proportion of the HmMalDH was found to be trapped as a stable complex with GroEL.

Because it is known that oligomeric chaperonin complexes assist the refolding and the release of their substrate in an ATP-dependent manner, we attempted to disrupt the complex by adding 5 mM MgATP. Because the GroES cochaperonin triggers the conformational changes of GroEL necessary for the release of the refolded protein (29), it was added in a stoichiometric amount in the experiments. We found that in the refolding experiments where MalDH was first mixed with the chaperone complexes at low salt (Fig. 6) or when the complexes were added to the hyposaline refolding buffer (Fig. 9A), the reactivation rates and the complex stability were not affected by the addition of MgATP (data not shown). We conclude that, in vitro, the P45 and GroEL systems can form complexes with denatured HmMalDH, but neither increases the productive refolding of the enzyme in hyposaline conditions.

DISCUSSION

P45, a Novel Ring-shaped ATPase—A novel 45-kDa protein (P45) was purified from the extreme halophilic archaeons H. marismortui and H. salinarium because P45 was abundant in a strain that could grow under the low salt concentrations known to represent stress conditions for halophilic proteins. It seems, however, that P45 overaccumulation is not an obligatory response because it was not observed systematically in strains that were adapted progressively to tolerate low salt concentrations. P45 displayed a ATPase activity that was found to be stronger in low salt conditions whereas the E. coli GroEL chaperonin was unaffected by the salt conditions. As for other halophilic enzymes, the maximum activity occurred...
just at the salt concentration (0.2 M) where fluorescence data showed that P45 starts to unfold (32, 41). The ATPase activity decreases with increasing salt. This is also a common trait for most halophilic proteins (2), and such a behavior has also been reported for a GroEL equivalent purified from a salt-tolerant bacterium (50). P45 shows no homology with any known chaperone. However, it exhibits a ring-shape oligomeric structure and ATPase activity that are hallmarks for molecular chaperones (42). This prompted us to examine the chaperonin function of P45.

**P45 Polypeptide Binding Activity**—Molecular chaperones are thought to play a cellular role in protecting mature proteins against denaturation and in assisting folding of nascent proteins (18, 51). Because the HmMalDH enzyme denatures upon incubation in low salt (0.5 M KCl) and refolds when placed back in hypersaline conditions (3 M KCl), we used it as a substrate for P45 in unfolding and refolding experiments. The *E. coli* chaperonin, GroEL, was also tested under the same conditions for its ability to assist the salt-dependent folding of a halophilic protein. We found that P45 recognizes and binds specifically to HmMalDH during denaturation induced by low salt stress or when the denatured protein is renatured in hypersaline conditions. This suggested that, similar to molecular chaperones, P45 binds to folding intermediates, which also explains the acceleration of the deactivation rates and the inhibition of the spontaneous refolding to the native state which were observed (52, 53). Similar effects were found with GroEL, indicating that a nonhalophilic chaperonin system can recognize the folding intermediates generated by salt stress. This supports the idea that the structural motifs responsible for the recognition of the substrate protein by molecular chaperones are not very specific and are generated whatever the denaturation process. It is believed that these structural motifs consist of hydrophobic patches exposed by native-like secondary structures (54).

**P45 ATP-dependent Chaperone Activity**—There is compel-
ling evidence that the binding of ATP weakens the affinity of chaperones for denatured protein substrates and facilitates the release of the substrate protein in an active native state (53, 55). The structure-function relationships of this process have been studied in detail for the E. coli chaperonin GroEL (9). ATP hydrolysis induces large conformational changes within the complexes, which facilitate the refolding of the bound misfolded polypeptides and their subsequent release in an active state (56, 57). If folding is incomplete, the same polypeptide may reenter the barrel, and the cycle continues until folding is complete. For most chaperones, this folding assistance functions during both denaturation and renaturation processes. P45 efficiently decreased the deactivation rate of HmMalDH in an ATP-dependent manner, which followed a first order reaction during the first 15 min of the incubation time. Thus, P45 was demonstrated to be a novel protein that catalyzes aggregation and denaturation when the salt concentration decreases in the cell environment as depicted in Fig. 10.

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