Uncoating of Coated Vesicles by Yeast hsp70 Proteins

(Received for publication, June 25, 1991)

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The ability of hsp70 isoenzymes from wild-type and mutant yeast strains to uncoat bovine brain coated vesicles was analyzed and compared with that of the brain uncoating ATPase. Results show that, among the four major cytoplasmic isoenzymes produced in wild-type yeast, almost all of the activity is associated with the SSA1 and SSA2 isoenzymes. The SSBl and SSB2 isoenzymes have almost no uncoating activity and are not found in the clathrin-hsp70 complexes formed during the uncoating reaction. Using hsp70 mutant yeast strains we find a marked difference in uncoating activity between the SSA1 and SSA2 isoenzymes, although there is only a 3% difference between their amino acid sequences. The SSA4 isoenzyme, which is produced only under stress conditions, has an uncoating activity intermediate between SSA1 and SSA2. These results suggest that the ability of hsp70 isoenzymes to uncoat clathrin-coated vesicles is restricted to certain members of the hsp70 family and can be affected by subtle changes in amino acid sequence. We also investigated the uncoating activity of mixtures of isoenzymes and find that the isoenzyme with lower uncoating activity reduces the activity of the isoenzyme with higher uncoating activity possibly by occupying binding sites on coated vesicles.

It has recently become clear that the bovine brain uncoating ATPase, which strips clathrin off of bovine brain clathrin-coated vesicles is a member of the hsp70 class of heat-shock proteins (Chappell et al., 1986; Ungewickell, 1985). This large class of proteins includes both proteins which are present constitutively in the cell and proteins which are induced during heat shock (for reviews, see Craig, 1985 and Lindquist, 1986). All of these proteins bind ATP very tightly, and one of their distinguishing properties is their ability to be isolated in an almost pure state with the use of an ATP-agarose affinity column.

Thus far, several rather diverse functions have been found for the hsp70 proteins, in addition to their ability to dissociate clathrin from coated vesicles. The most primitive of the hsp70 proteins, the dnaK protein of Escherichia coli, appears to be involved in numerous cellular functions as well as in bacteriophage replication (Phillips and Silhavy, 1990; Tilly and Yarmolinsky, 1989; Zylicz et al., 1983). Yeast has at least eight different hsp70 proteins (Lindquist, 1986), two of which have been shown to be involved in translocation of proteins across the membranes of endoplasmic reticulum (Chirico et al., 1988) and mitochondria (Deshaies et al., 1988), respectively. In mammalian cells, the hsp70 proteins appear to be involved in the uncoating process mentioned above, and, in addition, they appear to play a role in antigen presentation (Vanbuskirk et al., 1989) and nascent protein folding (Beckmann et al., 1990). Furthermore, BiP or GRP78, which is a member of this family located in the endoplasmic reticulum (Munro and Pelham, 1987), appears to be involved in complexing with aggregated proteins (Kozutsumi et al., 1988) and also in assisting multichain proteins to fold into correct quaternary structures (Bole et al., 1986; Flynn et al., 1989). All of these functions have been described under the umbrella term molecular chaperone (Ellis, 1987; Hemmingsen et al., 1988) suggesting that these proteins, in general, either fold, unfold, or otherwise alter the conformation of proteins to which they bind. For example, the role of the hsp70 proteins in translocation is thought to involve unfolding proteins so that they assume the correct conformation when they are presented to the machinery which is involved in transporting them across the membrane.

Recently, Flynn et al. (1989) found that both BiP and the uncoating ATPase are able to interact with a wide variety of peptides. Although this fits with the molecular chaperone idea, it does raise a question as to whether the hsp70 proteins show significant specificity in their interactions with various substrates. In the present study we investigated the ability of the cytoplasmic hsp70 proteins from yeast to dissociate clathrin from bovine brain coated vesicles. Using yeast mutants to produce specific hsp70 proteins (Craig and Jacobsen, 1984; Craig and Jacobsen, 1985), we find that these proteins have very different abilities to dissociate clathrin from coated vesicles. The SSBl and SSB2 isoenzymes apparently have almost no uncoating activity, while a mixture of SSA1 and SSA2 isoenzymes shows considerable uncoating activity, but less than that exhibited by the bovine brain uncoating ATPase. Furthermore, even the SSA1 and SSA2 isoenzymes, which differ only in a few amino acids, show a marked difference in their uncoating abilities, with the SSA2 isoenzyme approaching the activity of the bovine brain uncoating ATPase. Our results also suggest that the isoenzyme with lower uncoating activity may inhibit the activity of the isoenzyme with higher uncoating activity in a mixture of different isoenzymes possibly by occupying the binding sites on clathrin-coated vesicles.

MATERIALS AND METHODS

Purification of Bovine Brain Coated Vesicles and Uncoating ATPase—Coated vesicles were prepared from calf brain according to the procedure of Nandi et al. (1982). Generally, coated vesicles containing 60 mg of clathrin were obtained from about 2.5 kg of brain tissue (10 calf brains). The coated vesicles were stored at 4 °C at a clathrin triexkilon concentration of about 15 μM. Bovine brain uncoating ATPase was purified by the method of Schlossman et al. (1984) with slight modifications as described previously (Greene and Eisenberg, 1990).

Purification of Yeast hsp70 Proteins—The yeast strains used were
developed in the laboratory of Dr. E. Craig at the University of Wisconsin. All steps in the yeast hsp70 protein preparation were carried out at 0-4 °C unless otherwise indicated. Cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) until the A<sub>600</sub> of the culture reached 6 at which point they were harvested by Millipore filtration. The cell paste was then dropped into liquid nitrogen and stored at -70 °C until use. In a typical preparation, 60 g of the cell paste were suspended in 180 ml of homogenization buffer (buffer A: 40 mM imidazole, 75 mM KCl, 5 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μM leupeptin, 10 μM pepstatin A, pH 7.0) and mixed with 240 g of glass beads (10 mm in diameter, from B. Braun Melseeugen AG). The cells were broken using a Biospec bead-beater (model 909-1) with four to five cycles of 2-min beating and 10-min sitting. A Zeiss light microscope was used to determine that over 90% of the cells were broken. The cell homogenate was then centrifuged at 5,000 rpm for 10 min in a Sorvall SS34 rotor. The supernatant was centrifuged again at 34,000 rpm for 150 min in a Beckman Ti-50.2 rotor. The resulting supernatant (normally about 130 ml, with a protein concentration of 5 mg/ml) was then loaded onto a 5-ml ATP-agarose (Sigma, Cat. A2767) column equilibrated with elution buffer (buffer C: 20 mM imidazole, 25 mM KCl, 10 mM ammonium sulfate, 2 mM magnesium acetate, 1 mM dithiothreitol, pH 7.0) and mixed with 240 g of glass beads (6.0 mm in diameter, from B. Braun Melseeugen AG). The column was then washed with 30 ml of buffer C, 40 ml of buffer C containing 1 M NaCl, and 30 ml of buffer C again, and finally, eluted with buffer C containing 1 mM ATP. The yeast hsp70 protein was precipitated by addition of ammonium sulfate to 70% saturation to the ATP-eluted fraction, and the precipitate was collected by a 20-min centrifugation at 18,000 rpm in a Sorvall SS34 rotor. The protein was dialyzed against buffer C overnight before use. This preparation method yielded hsp70 protein which was at least 90% pure determined by densitometric scanning of the SDS<sub>1</sub> gels. The hsp70 protein concentration was determined using its extinction coefficient (ε<sub>280 nm</sub> = 4.5, molecular mass = 70 kDa) which had previously been determined by amino acid composition analysis.

Assay for Coated Vessel Uncoating Activity—The uncoating activity of the yeast hsp70 protein was measured as described by Greene and Eisenberg (1990). Briefly, the coated vesicles were mixed with hsp70 protein in the presence of MgATP and incubated at 25 °C. The reaction mixture was then centrifuged in a Beckman TL-100 centrifuge to pellet the remaining coated vesicles. The supernatant, containing soluble hsp70 protein and clathrin released from coated vesicles, was analyzed by SDS gel. The Coomassie Blue-stained hsp70 protein and clathrin bands were quantified using an LKB Ultrascan XL laser densitometer.

FPLC Column Chromatography—FPLC was performed using a Pharmacia LKB Biotechnology Inc. Superose-6 column (HR 16/50) and a Pharmacia FPLC system with Gp-250 programmer, P-500 pumps, Single-Path UV-1 monitor, and Frac-100 fraction collector. The column was equilibrated in buffer C, pH 7.0. For separating the upper and lower bands, 2 ml of purified hsp70 protein at a concentration of 15 μM were loaded onto the column. For separating clathrin and clathrin-hsp70 complexes from free enzyme in the uncoating supernatant, 2 ml of the supernatant of a uncoating reaction mixture with 10 μM hsp70 protein and coated vesicles containing 1 μM clathrin triskelions were loaded onto the column. The column was run at a flow rate of 1.5 ml/min. Fractions of 1 ml were collected and analyzed by SDS-polyacrylamide gel electrophoresis.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (1975) using amfonilines from Bio-Rad (1.6%, pH 5-7; 0.4%, pH 3-10).

RESULTS

Purification of Yeast hsp70 Protein—A high degree of purification of hsp70 proteins from yeast can be obtained simply by chromatography of crude lysates on an ATP-agarose column (Fig. 1). After elution from the ATP-agarose column, we obtained a doublet at approximately 70 kDa with an upper to lower band ratio of 3 to 2 in stain intensity (lane 4). Since we were purifying the hsp70 proteins from wild-type yeast, which contains a number of hsps, this doublet may be due to the presence of a mixture of isoenzymes in our final preparation.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography.

Fig. 1. Purification of yeast hsp70 protein. Purified hsp70 protein (lane 4) from wild-type yeast consists of two bands on the one-dimensional SDS gel. The ratio of the upper band to the lower band is 3:2 by densitometric scanning. Lane 1, high speed supernatant of yeast homogenate (see “Materials and Methods”); lane 2, flow-through of the ATP-agarose column; lane 3, salt-wash fraction of the column; lane 4, ATP-eluted purified yeast hsp70 protein; and lane 5, purified bovine brain uncoating ATPase.

Fig. 2. Comparison of the uncoating activity of the yeast hsp70 protein and brain uncoating ATPase. The uncoating of the brain coated vesicles by yeast hsp70 protein and brain uncoating ATPase was carried out in buffer C (see “Materials and Methods”) with an ATP regenerating system (30 units/ml creatine phosphokinase and 15 mM phosphocreatine from Sigma) at 25 °C. The enzyme concentration for both yeast hsp70 protein and brain uncoating ATPase is 0.8 μM. The total coated vesicles used contained 0.5 μM clathrin triskelions. The amount of clathrin in the uncoating supernatant was determined by densitometric scanning of SDS gels. ○, uncoating by yeast hsp70 protein; ●, uncoating by bovine brain uncoating ATPase. The initial burst of uncoating was estimated by extrapolation of the linear steady-state rate (dashed lines).

For comparison the bovine brain uncoating ATPase is shown in lane 5. Densitometric scanning of the SDS gel showed that the yeast hsp70 protein is more than 90% pure.

Uncoating Activity of the Yeast hsp70 Protein—We next tested the ability of the purified wild-type yeast hsp70 protein preparation to release clathrin from bovine brain coated vesicles. Using brain uncoating ATPase, we had previously found that there was an initial burst of uncoating followed by slow steady-state uncoating activity (Greene and Eisenberg, 1990). Fig. 2 shows a comparison of the time course of uncoating activity by the yeast hsp70 proteins prepared from wild-type yeast and the brain uncoating ATPase. Like the brain uncoating ATPase, the yeast protein showed an initial burst of uncoating followed by slow steady-state uncoating activity. However, the magnitude of the initial burst of uncoating and the steady-state rate of uncoating were only about 40 and 25%, respectively, of the values observed with the brain enzyme.

Fig. 3 shows the magnitude of the initial burst of uncoating as a function of enzyme concentration with both wild-type yeast hsp70 protein and brain uncoating ATPase. As we found previously with brain uncoating ATPase, the initial burst of
Uncoating Activity of Yeast hsp70

Uncoating was stoichiometric with three enzyme molecules dissociating one clathrin molecule. However, with yeast hsp70 protein, the initial burst of uncoating was markedly decreased with considerably more enzyme required to dissociate the clathrin from the coated vesicles in the initial burst. Furthermore, even at a ratio of hsp70 enzyme uncoating ATPase to clathrin much higher than the stoichiometric binding ratio, the yeast hsp70 protein did not release clathrin from coated vesicles as extensively as did stoichiometric amounts of brain enzyme.

Separation of the Upper and Lower hsp70 Bands by FPLC—One reason that the wild-type yeast hsp70 preparation was less effective than the brain enzyme may be that it is a mixture of various isoenzymes of yeast which have different levels of uncoating activity. We, therefore, attempted to determine the activity of the individual isoenzymes present in the wild-type yeast preparation. We began by attempting to separate the wild-type hsp70 proteins on a Superose-6 column using FPLC. Fig. 4 shows that the upper and lower bands observed on a one-dimensional SDS gel are partially separated by chromatography on FPLC. The upper band elutes first followed by the lower band.

We next tested the different fractions from the FPLC column for uncoating activity (Fig. 5, upper panel). Qualitatively, it is clear that much more clathrin was released by the earlier column fractions, which contained mostly the upper band, than by the later fractions. The amount of dissociated clathrin as a function of the protein in the upper and lower bands was quantified by densitometry (Fig. 5, lower panel). It is clear that almost all of the uncoating activity was associated with the upper band on the one-dimensional gel, but the lower band showed little or no uncoating activity.

To determine which yeast isoenzymes were associated with the upper and lower bands, we compared a two-dimensional gel of the wild-type enzyme with two-dimensional gels of the upper and lower bands separated by the FPLC column. Fig. 6a shows that the hsp70 that we originally isolated from wild-type yeast was mainly a mixture of SSA1 and SSA2 isoenzymes and SSB1 and SSB2 isoenzymes. These isoenzymes were originally identified by analysis of strains containing single mutations (Chappell et al., 1986; Craig and Jacobsen, 1984; Craig and Jacobsen, 1985). Fig. 6b shows that the upper band from the SDS gel was composed mainly of the SSA1 and SSA2 isoenzymes, while the lower band (Fig. 6c) was composed mainly of the SSB1 and SSB2 isoenzymes. Therefore, most of the uncoating activity observed with the wild-type yeast appeared to be associated with the SSA1 and SSA2 isoenzymes with almost no activity associated with the SSB1 and SSB2 isoenzymes.

Isolation of Clathrin-Enzyme Complexes Formed during Uncoating—Following the uncoating reaction, brain uncoating ATPase forms a long-lived complex with clathrin (Schmid...
and Rothman, 1985; Schmid et al., 1985); about 50-60% of the free clathrin is complexed with enzyme following separation of free enzyme from uncoated clathrin and its associated bound enzyme on an FPLC column (data not shown). If indeed the SSB1 and SSB2 isoenzymes have very little activity, it might be expected that, following the uncoating reaction with wild-type enzyme, considerably less of these isoenzymes would bind to clathrin than the SSA1 and SSA2 isoenzymes. To test this, we used FPLC to separate the free enzyme from the uncoated clathrin and its associated bound enzyme. With the wild-type yeast enzyme the clathrin was found to be about 35% saturated with enzyme; somewhat less complex than was observed with the brain uncoating ATPase. Fig. 7a shows an FPLC chromatogram of the uncoating supernatant of wild-type hsp70 and Fig. 7b shows two-dimensional gels of wild-type hsp70 proteins and isoenzymes found associated with clathrin during uncoating. As can be seen, the SSA1 and SSA2 isoenzymes, but almost no SSB1 and SSB2 isoenzymes were complexed with clathrin. Interestingly, the two-dimensional gels in Fig. 7b suggest that a little more SSA2 than SSA1 isoenzyme was complexed with clathrin compared to the ratio found in the purified wild-type hsp70 protein. This suggests that the SSA2 isoenzyme may have a slightly higher activity than the SSA1 isoenzyme.

Since it was possible that the SSB isoenzymes bound to the coated vesicles in a dead-end complex even though they were unable to uncoat clathrin from coated vesicles, we also analyzed the uncoating pellet which contains the coated vesicles which sediment following the uncoating reaction. We found that the clathrin was 30% saturated with SSA1 and SSA2, but no SSB1 and SSB2 isoenzymes bound to the pelleted coated vesicles (Fig. 8). These results are consistent with almost all of the uncoating activity observed with wild-type enzyme most likely being carried out by the SSA1 and SSA2 isoenzymes. Since the SSB1 and SSB2 isoenzymes neither bind to the dissociated clathrin or to the coated vesicles (Fig. 7b and 8), these results strongly suggest that the presence of SSB1 and SSB2 isoenzymes has no effect on the uncoating reaction carried out by SSA1 and SSA2.

Uncoating Activity of Different hsp70 Isoenzymes—Since the SSB1 and SSB2 isoenzymes make up about 40% of the mixture of wild-type enzymes, the fact that they show almost no activity partially explains our observation that the wild-type enzyme was not as active as the brain uncoating ATPase. However, even taking this into consideration, the yeast enzyme still showed considerably less activity than the brain uncoating ATPase. Therefore, we wondered whether there...
was also a difference in the activity of the SSA1 and SSA2 isoenzymes. It might be expected that these two isoenzymes would have about the same activity since there is only a 2% difference in their amino acid sequences (Slater and Craig, 1989a). However, their regulatory sequences are quite different (Slater and Craig, 1989a) suggesting that they may have different functions in the yeast cell. Therefore, using yeast mutants which lack either the SSA1 or SSA2 isoenzyme we tested the activity of these individual isoenzymes.

Fig. 9 shows the uncoating activity of the isoenzymes purified from ssal mutant (ssal-) or ssab mutant (ssab-). For comparison we also show the activity of the wild-type enzyme and the brain uncoating ATPase. Note that the preparations of purified SSA1 and SSA2 isoenzymes did contain the SSB isoenzymes, but based on the results presented above, we would expect that they have no effect on the observed uncoating activity. Therefore, the activity of the yeast enzymes are plotted as a function of the amount of upper band on one-dimensional SDS gel, i.e. on the amount of SSA1 and/or SSA2 isoenzyme present. Surprisingly, despite their similar amino acid sequences there was a marked difference in the activity of the SSA1 and SSA2 isoenzymes. The activity of the SSA2 isoenzyme approached that of the brain uncoating ATPase while the SSA1 isoenzyme had less activity than the SSA2 isoenzyme. The activity of the SSA1 and SSA2 isoenzymes did not dissociate as much clathrin from coated vesicles as the SSA2 isoenzyme.

Interestingly, these data suggest that the activity of the wild-type enzyme is not just the sum of the activities of the SSA1 and SSA2 isoenzymes. If this were the case, when the wild-type enzyme was twice the concentration of the SSA2 isoenzyme, the activity of the wild-type enzyme should be equal to or greater than the activity of the SSA2 isoenzyme. This is because nearly equal amounts of SSA1 and SSA2 isoenzymes are present in preparations from wild-type yeast (Figs. 6a and 7b). However, at high enzyme concentration where the activity of the all of the yeast enzymes levels off, the activity of the wild-type enzyme (solid circles, Fig. 9) was, in fact, significantly less than the activity of SSA2 isoenzyme (open triangles, Fig. 9) at half of the concentration. An identical result was obtained if a mixture of purified isoenzymes from ssal- and ssab- strains was used (open circles, Fig. 9) instead of wild-type enzyme. This suggests that in addition to the SSA1 isoenzyme having less activity than the SSA2 isoenzyme, it may actually inhibit the activity of the SSA2 isoenzyme. To further investigate this phenomenon, we studied the effect of the yeast isoenzymes on the activity of the bovine brain uncoating ATPase. As can be seen in Table I, both with SSA1 and SSA2, mixtures of the yeast isoenzymes with the uncoating ATPase show considerably less than additive uncoating activity. It is possible that this effect is due to a competition between the yeast isoenzymes and the uncoating ATPase for binding sites on the coated vesicles. The binding of the yeast enzymes may compete with the binding of the more active uncoating ATPase thus reducing the uncoating activity which occurs. A similar effect may also explain why the SSA1 and SSA2 isoenzymes do not exhibit additive uncoating activity.

To assess more fully the differences in activity among the different hsp70 isoenzymes, we isolated hsp70 isoenzymes from different mutant strains. The two-dimensional gels in Fig. 10 show the hsp70 isoenzymes present in different yeast mutant strains, and Table II shows the activity of these different preparations. In each case we have presented the activity per amount of upper band of the isoenzyme. The ssal-ssab double mutant (DS16) produces SSA1 isoenzyme (Craig and Jacobsen, 1984), which is not expressed in wild-type yeast.

![Fig. 9. Comparison of the uncoating activity of SSA1 and SSA2 yeast isoenzymes.](image)

![Fig. 10. Two-dimensional gels of hsp70 proteins isolated from different yeast strains.](image)

**TABLE I**

| Coated vesicles | Isoenzymes from | 0.18 | 0.32 | 0.32 |
|-----------------|-----------------|------|------|------|
| 2 μM yeast enzyme | ssal- | 0.09 | 0.11 | 0.19 |
| 2 μM yeast enzyme and 0.6 μM uncoating ATPase | ssal- | 0.17 | 0.19 | 0.19 |
| 2 μM yeast enzyme and 0.6 μM uncoating ATPase | ssab- | 0.12 | 0.17 | 0.21 |

**FIG. 9.** Comparison of the uncoating activity of SSA1 and SSA2 yeast isoenzymes. The initial burst of uncoating by the isoenzymes isolated from wild-type yeast (●), ssal mutant (● ssal-), ssab mutant (● ssab-), and brain uncoating ATPase (●) was measured as in Fig. 3. The initial burst of uncoating by the mixture of equal amount of isoenzymes from ssal- and ssab- strains are indicated by ○.
Lower band SSA4 SSA1 SSA2 SSA1 SSA1

and/or nucleotide and therefore lower the uncoating activity of SSA1 compared to SSA2. Flynn et al. (1989) recently demonstrated that a large number of rather nonspecific peptides bind to the constitutive mammalian hsp70 protein which raises the question of how specifically the hsp70 proteins interact with their various substrates. The data presented in this paper suggest that, at least in regard to the uncoating reaction, the interaction appears to be quite specific. Not only did the various isoforms of the hsp70 proteins differ markedly in their ability to uncoat clathrin-coated vesicles but in addition subtle differences in amino acid sequence had a significant effect on uncoating activity. This is of particular interest, because up to the present time the most clearly defined activity of the constitutive mammalian hsp70 protein is its ability to strip clathrin off of coated vesicles. We conclude from these data that the ability to uncoat bovine brain coated vesicles is specific to certain members of the hsp70 family and depends on subtle differences in amino acid sequence. In addition, these data suggest that it is the SSA isoenzymes from yeast which are most similar to the bovine brain uncoating ATPase with the SSA2 isoenzyme approaching the bovine brain uncoating ATPase in activity.

One question which arises from this work is whether SSA2, or any of the other yeast 70,000 heat-shock proteins, are actually involved in uncoating clathrin-coated vesicles in yeast. One reason that the SSA2 isoenzyme may not be as active as the brain enzyme is that we are not using yeast clathrin-coated vesicles as substrate. In fact, there is evidence that clathrin-coated vesicles occur in yeast and, if they occur, presumably at some point they must be uncoated. In this regard, it is of interest that SSA2 isoenzyme is the only SSA protein which is entirely constitutive; presumably the uncoating of clathrin-coated vesicles is a constitutive process in yeast. However, at the present time, there is no evidence as to whether SSA2 isoenzyme or the other yeast hsp70 proteins are involved in this process. The only defined function for the yeast hsp70 proteins is their role in the translocation of proteins into the endoplasmic reticulum (Chirico et al., 1988) and mitochondria (Deshai et al., 1988). Given the relationships between the mammalian uncoating ATPase and the SSA proteins in yeast it will be of interest to determine if the mammalian protein can replace the yeast protein in this function in vivo.

One other interesting point noted in these studies is that, when mixtures of isoenzymes are used, the isoenzyme with lower activity can actually inhibit the activity of the isoenzyme with higher activity. This is true both for mixtures of SSA1 with SSA2 and for mixtures of either SSA1 or SSA2 with bovine brain uncoating ATPase. It may be related to the observation that, with the yeast enzymes, complete uncoating of the coated vesicles is not observed even at very high concentrations of enzyme. Although it is not clear why complete uncoating does not occur, it may be due to the fact that some of the coated vesicles are so stable that they cannot be uncoated by the less active enzyme during the initial burst of uncoating. However, if the less active enzyme binds to these stable coated vesicles and prevents the more active enzyme from uncoating them, it could explain why the presence of less active enzyme inhibits the activity of more active enzyme.

We are currently studying the binding of the uncoating ATPase to clathrin under various conditions, and it is possible that these studies will shed light on whether a dead-end complex can, in fact, form between the yeast isoenzyme and the clathrin-coated vesicles.

Acknowledgment—We thank Angela Murphy for running the amino acid analysis of the yeast hsp70 protein.

Eight different hsp70 proteins, including BiP (Normington et al., 1989; Rose et al., 1989) have been isolated from yeast. In the present study we tested the ability of five of the six cytoplasmic proteins to uncoat clathrin-coated vesicles. Our results show that, while the SSA1, SSA2, and SSA4 isoenzymes are able to uncoat bovine brain coated vesicles, the SSB1 and SSB2 isoenzymes, which are 65–75% similar in amino acid sequence to the SSA isoenzymes (Slater and Craig, 1989b), are unable to carry out significant uncoating. Furthermore, they do not appear to bind to clathrin dissociated from coated vesicles or to the coated vesicles themselves. Therefore, although our preparations of SSA1, SSA2, and SSA4 isoenzymes are contaminated with the SSB isoenzymes, we consider it very unlikely that they are affecting the uncoating activity which we observe. Furthermore, since our two-dimensional gels suggest that SSc1 and BiP are only present in very small amounts in our preparations and Ssa3 is not expressed, we think it likely that the preparations of SSA1, SSA2, and SSA4 isoenzymes which we prepared from the mutant yeast strains are acting as relatively pure preparations of a single isoenzyme.

Not only is there a major difference in the uncoating activity of the SSA and SSB isoenzymes, but, in addition, the SSA1 isoenzyme shows considerably less activity than the SSA2 isoenzyme although they differ in only a few amino acids. Comparing the amino acid sequences of SSA1 and SSA2 isoenzymes, Slater and Craig have shown that SSA1 has four amino acid substitutions at the N-terminal end, which is close to the putative nucleotide binding site, and 11 amino acid substitutions in the rest of the molecule with a three-amino acid insertion at the C-terminal end, which is presumably where clathrin binds (Slater and Craig, 1989a). Furthermore, six of the 15 amino acid substitutions are conservative in nature.

It is remarkable that this small number of changes is apparently sufficient to alter the way SSA2 binds clathrin except under stress conditions. SSA4 isoenzyme, which is probably produced to compensate for the loss of the SSA1 and SSA2 isoenzymes, migrates as an upper band on one-dimensional gels like the SSA1 and SSA2 isoenzymes. The uncoating activity of the SSA4 isoenzyme is intermediate between the SSA1 and SSA2 isoenzymes. Interestingly, the mutant which is lacking the SSB1 and SSB2 isoenzymes (T5151) had significantly lower activity than the wild-type enzyme. The two-dimensional gel of the hsp70 proteins isolated from this yeast strain indicates that it had considerably more SSA1 than SSA2 isoenzyme (Fig. 10), in contrast to the wild-type yeast which had approximately equal amounts of the two isoenzymes. This may account for the lower activity of the enzyme isolated from this strain of yeast compared to the wild-type enzyme.

**DISCUSSION**

| TABLE II     | Uncoating activity of different hsp70 proteins |
|--------------|-----------------------------------------------|
| Strains      | SSA1 | SSA2 | SSA4 | SSA1 | SSA2 |
| DS10 (wild-type) | SSA1 | SSA2 | SSA4 | SSA1 | SSA2 |
| DS16 (SSAZ)  | SSA1 | SSA2 | SSA4 | SSA1 | SSA2 |
| T5151        | SSA1 | SSA2 | SSA4 | SSA1 | SSA2 |
| MW118        | SSA1 | SSA2 | SSA4 | SSA1 | SSA2 |
| MW120 (SSAZ) | SSA1 | SSA2 | SSA4 | SSA1 | SSA2 |

*Uncoating mixture had coated vesicles containing 1 μM clathrin triskelions, and 0–3 μM (upper band) yeast hsp70 proteins. Values are averages of five independent experiments and presented as μM clathrin released per μM of upper band isoenzymes present (assuming SSB1 and SSB2 have no uncoating activity).

Values are averages of five independent experiments and presented as μM clathrin released per μM of upper band isoenzymes present (assuming SSB1 and SSB2 have no uncoating activity).
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