Heat Shock Protein 80 of *Neurospora crassa*, a Cytosolic Molecular Chaperone of the Eukaryotic Stress 90 Family, Interacts Directly with Heat Shock Protein 70†

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ABSTRACT: The subunit structure of Hsp80, the most abundant heat-shock protein of *Neurospora crassa*, was examined by chemical cross-linking of the purified protein *in vitro*. Resolution of glutaraldehyde-treated Hsp80 by sodium dodecyl sulfate−polyacrylamide gel electrophoresis SDS−PAGE suggests that the native state of this protein is a tetramer; the relative proportion of cross-linked species, estimated by the fraction of protein recovered in each category, is consistent with a dimer-of-dimer structure. Upon interaction with nucleotides, higher order cross-linked oligomers were detected, indicating ligand-induced conformational changes. The effect of nucleotides was also monitored by following tryptophan fluorescence: CTP, UTP, and NAD led to fluorescence quenching, the effect of CTP being the most pronounced. As individual molecular chaperones often act in concert with cochaperones, interaction between the two major cytosolic stress proteins—Hsp80 and Hsp70—was examined. Purified Hsp70 was immobilized on ATP−agarose and purified Hsp80 was applied to the Hsp70-saturated matrix; while Hsp80 did not bind to ATP−agarose by itself, it was bound strongly by immobilized Hsp70. The [Hsp70−Hsp80] complex was eluted with ATP and coelution of both proteins was confirmed by Western blot analysis, using specific polyclonal antibodies raised against each protein. The physical association of stress-inducible Hsp70 and Hsp80 was verified by interprotein cross-linking *in vitro* followed by immunoblot analysis and by immunoprecipitation.

Exposure of cells and organisms to adverse physiological conditions, such as hyperthermia, oxidative stress, toxic chemicals, metabolic poisons, viral infection, and certain disease states, triggers the accelerated synthesis of defense-related proteins, commonly described as heat-shock proteins (Hsps) or simply as stress proteins [reviewed in Craig et al. 1993)]. Many of the Hsps are produced under normal growth conditions as well, but in the face of unfavorable circumstances their rate of synthesis is enhanced substantially. Hsps and their normal cellular counterparts, or cognates, perform vital functions as molecular chaperones. The latter group comprises a class of proteins, responsible for regulating the formation of the biologically active structure of proteins, by promoting requisite folding and maturation of cellular polypeptides during normal growth. Under stress conditions, however, they suppress protein aggregation, denaturation, and misfolding and, in addition, catalyze degradation of aggregated/denatured or otherwise structurally aberrant proteins [reviewed in Gething Sambrook (1992) and Hartl (1996)]. Hyperthermal exposure results in an increased accumulation of denatured proteins; hence the necessity for their rapid removal or efficient repair of the damage incurred thereby.

The best-studied examples of molecular chaperones belong to three major stress-protein families: eukaryotic Hsp70 and cognates and the prokaryotic analog, DnaK; the GroEL/ES chaperonin family of bacterial, mitochondrial, and chloroplast origin (Hartl, 1996); and the eukaryotic stress 90 proteins, frequently acting in concert with other cytosolic proteins (Jacob & Buchner, 1994). Members of the mammalian stress 70 family are known to exhibit molecular chaperone activity by virtue of an ATP-dependent interaction, preferentially, with hydrophobic regions of nascent polypeptides in the cytosol (Beckman & Mizzen, 1990).

The stress 90 family members, on the other hand, have been proposed to function as general chaperones, presumably in an ATP-independent manner, in close association with a repertoire of cytosolic protein cofactors (Jacob & Buchner, 1994). Their role in suppression of aggregation of unfolded proteins and promotion of refolding of unfolded polypeptides *in vitro* has also been established (Wiech et al., 1992; Shaknovich et al., 1992). The mammalian Hsp90 has often been observed in complexes with a variety of other proteins in the form of multiprotein assemblies, some notable examples being mammalian steroid receptors (Pratt, 1993), the aryl hydrocarbon, Ah, receptor in yeast (Carver et al., 1994) calmodulin (Minami et al., 1993), protein kinases (Xu & Lindquist, 1993; Aligue et al., 1994), and the oncogenic viral protein v-Src, a nonreceptor tyrosine kinase (Brugge et al., 1981), where Hsp90 has been shown to be a critical factor implicated in maturation of substrate proteins.

Hsp90 has been encountered in multicomponent complexes, in association with steroid hormone receptors along with the immunophilin FKBP52 (also described as Hsp56), where it is thought to recognize a defined feature of the

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substrate, namely, the tetratricopeptide domain (Goebl & Yanagida, 1991; Owens-Grillo et al., 1996). Interestingly, within the multimeric assembly containing the steroid receptors, both Hsp90 and Hsp70 are present together; the multicomponent progesterone receptor complexes, assembled *in vitro*, also contain these two molecular chaperones but appear to recruit an additional component, p60, possibly as a linker between Hsp70 and Hsp90 (Chen et al., 1996). In such systems, Hsp70 and 90 have been proposed to act synergistically in catalyzing the conversion of the biologically inactive form of the receptor to its active, steroid-binding conformation. However, in these complexes the relationship between Hsp70 and Hsp90 is not clearly understood and in no instance has a direct interaction between Hsp70 and Hsp90 been demonstrated.

A variety of stress treatments induce rapid and transient synthesis of several proteins in the filamentous fungus *Neurospora crassa*, including Hsp70 and Hsp80, the major heat-inducible members of the stress 70 and 90 families, respectively (Kapoor & Lewis, 1987). In *N. crassa* Hsp80 is the most abundant stress protein—synthesized in response to heat shock and carbon source starvation—with a predominantly cytosolic distribution, as witnessed by immunoelectron microscopy of gold-labeled thin sections (Roychowdhury et al., 1992a). The gene encoding this protein was cloned and the sequence deduced from the cDNA was shown to exhibit marked homology to several of the eukaryotic stress 90 family members. The C-terminal end of the polypeptide has the sequence motif MEEVD, also found in several stress 90 homologues, characteristic of the cytosolic members of this group (Roychowdhury et al., 1992b). The presence of a IEEVD sequence at the C-terminus of the major member of the *N. crassa* Hsp70 family is also evident in the amino acid sequence derived from the nucleotide sequence of the cloned gene (Kapoor et al., 1995). In view of its abundance in heat-shocked cells and similarity to the mammalian stress 90 family members, *N. crassa* Hsp80 provides an excellent model for studies of structure−function relations and interprotein interactions of the Hsp90 family members.

This paper contains information on the purification and subunit structure of Hsp80 and data on its interaction with Hsp70. Experiments with chemical cross-linking and Western blot analysis showed Hsp80 to be a multimer, most likely a tetramer, in its native state. Interaction with nucleotide triphosphates resulted in the formation of higher order oligomeric units and in quenching of intrinsic tryptophan fluorescence, suggesting the propagation of conformational changes by these ligands. Evidence for a direct interaction between Hsp80 and Hsp70 was sought by interprotein cross-linking, affinity chromatography, and immunoblot analysis of cross-linked products with polyclonal antibodies raised against both of these proteins. Demonstration of a direct physical interaction between Hsp80 and Hsp70, the formation of homooligomers of Hsp80 and heterooligomers of Hsp70 and Hsp80, is presented.

MATERIALS AND METHODS

Growth of Cultures and Heat-Shock Treatment. *N. crassa* wild-type strain (Fungal Genetics Stock Center no. 262) was grown for 15 h, in Vogel's minimal medium, Vm (Vogel, 1956) with 2% sucrose as the carbon source, while shaking at 28 °C. Heat shock was administered by transferring 15-h-old cultures to 48 °C for 1 h (Kapoor & Lewis, 1987). The mycelium was harvested by vacuum filtration, lyophilized, and stored at −20 °C.

Purification of Hsp80. For purification of Hsp80, heat-shocked, lyophilized mycelium was suspended in 10 volumes of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 1 mM β-mercaptoethanol and stirred at 4 °C for 40 min. The slurry was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 12000*g* for 20 min. The supernatant was subjected to ammonium sulfate precipitation and the fraction, enriched in Hsp80, precipitating between 50% and 70% saturation was dissolved in a small volume of 20 mM Tris-HCl (pH 7.5) and dialyzed overnight against several changes of the same buffer. Fractions from this and subsequent steps in the purification procedure were monitored for the presence of Hsp80 by electrophoretic resolution in SDS−polyacrylamide minigels (Mini Protean II; Bio-Rad) followed by Western blot analysis employing a specific polyclonal anti-Hsp80 IgG prepared against *N. crassa* Hsp80.

The dialyzed, 50−70% ammonium sulfate fraction was applied to a 10-mL concanavalin A−Sepharose 4B (Pharmacia) column. Elution was performed with 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 1 mM each $CaCl_2$, $MnCl_2$, and $MgCl_2$. Fractions of the eluent enriched in Hsp80 were pooled and applied to a 10-mL Cibacron Blue−agarose (Sigma) column, previously equilibrated against 20 mM Tris-HCl (pH 7.5). Elution was carried out with a linear gradient of 0−1.75 M NaCl in the same buffer, 2-mL fractions were collected, and those enriched in Hsp80 were pooled and precipitated with 80% saturation of ammonium sulfate, the precipitate being dissolved in 20 mM Tris-HCl (pH 7.5) buffer and dialyzed against four changes of the same buffer at 4 °C. The dialyzed sample was chromatographed through a Mono Q anion-exchange column (HR 5/5) attached to a Pharmacia fast protein liquid chromatography (FPLC) system that had been preequilibrated with the dialysis buffer. The protein sample (0.9 mL) was clarified by centrifugation, loaded onto the column, and eluted with a discontinuous NaCl gradient, prepared in the same buffer. The elution gradient steps consisted of 0−0.3 M (7.5 mL), 0.3−0.44 M (22 mL), and 0.44−1.0 M NaCl (7.5 mL). One-milliliter fractions were collected and tested for Hsp80 status by Western blot analysis.

Alternatively, prior to the Mono Q step, fractionation was carried out using an octyl-Sepharose column (Pharmacia), instead of Con A−Sepharose. The sample was applied (in 30% saturated ammonium sulfate), to the column that had been preequilibrated with 20 mM Tris-HCl (pH 7.5) containing 30% saturated ammonium sulfate. The column was washed with the latter buffer containing 30% and 5% saturated ammonium sulfate, in succession, and finally the protein was collected by elution with the original buffer lacking ammonium sulfate. The fractions containing Hsp80 were pooled and concentrated using Centricon filter concentrators (30 kDa cutoff).

Purification of Hsp70. A crude extract of lyophilized, heat-shocked mycelium was prepared as described in the preceding section. The following procedure used by us is a modification of that employed by Fracella et al. (1993), based on the previously published method of Welch and Feramisco (1985). The protein precipitating between 40% and 70% saturation of ammonium sulfate was dissolved in 10 mM

Tris-HCl (pH 7.5), 10 mM NaCl, 20 mM $MgCl_2$, and 1 mM PMSF (phenylmethanesulfonyl fluoride) and dialyzed overnight against the same buffer lacking PMSF. The solution was clarified by centrifugation at 12000*g* for 20 min; the supernatant was recentrifuged at 48000*g* for 1 h. The supernatant from the last step was loaded on a DEAE-cellulose (Sigma) column equilibrated against the same buffer. Elution was conducted using a linear 0−1.0 M NaCl gradient, and the resulting fractions were monitored for the presence of Hsp70 by electrophoresis in 10% polyacrylamide, isocratic denaturing gels (Laemmli, 1970). The peak fractions containing Hsp70 were combined and applied to a 1-mL ATP−agarose (Sigma) column. The column was washed with the loading buffer, followed by 0.6 M NaCl in the loading buffer to remove nonspecifically bound proteins. After thorough washing with the loading buffer to remove NaCl, Hsp70 was eluted with 5 mM ATP in the loading buffer, all of the protein being recovered in two 1-mL fractions. The major component in the eluate was Hsp70 along with another protein, migrating to a position corresponding to approximately 40 kDa in denaturing gels. The eluted material was concentrated using Centricon filter units (30 kDa cutoff) and Hsp70 was separated from the 40-kDa component by gel filtration using a Superose-12 (Pharmacia) column linked to an FPLC unit.

Chemical Cross-Linking, SDS−PAGE, and Western Blotting. Intersubunit cross-linking of Hsp80 was performed by means of glutaraldehyde. Reaction mixtures containing 10−20 μg of Hsp80 in 20 mM Tris-HCl (pH 7.5) in a total volume of 30 μL were treated with 2 μL of 2.3% freshly prepared glutaraldehyde and incubated at 37 °C for 2 min. The reaction was terminated by addition of 5 μL of 1 M Tris-HCl, pH 8.0. Cross-linked proteins were solubilized by addition of 10 μL of Laemmli sample buffer (Laemmli, 1970) to which 2 μL of 0.1% bromophenol blue was added, and electrophoresis was conducted in step-gradient SDS-polyacrylamide gels consisting of 5%, 10%, 12.5%, and 15% polyacrylamide layers, using the Bio-Rad Mini Protean II apparatus. Proteins were visualized by staining the gels with Coomassie blue. For Western blotting, proteins resolved by SDS−PAGE were electroblotted onto nitrocellulose membranes at 4 °C, for 13 h, at 20 V. The immunoblots were probed with a 1:50 000 dilution of anti-Hsp80 IgG and 1:10 000 dilution of the secondary antibody (goat anti-rabbit horseradish peroxidase conjugate; Sigma). The bands were visualized by treating the immunoblots with the ECL chemiluminescence reagents (Amersham), according to the supplier's instructions, followed by exposure to X-ray films (Kodak X-Omat or equivalent).

Fluorescence Measurements. Interaction of various nucleotides with Hsp80 was assessed by monitoring changes in intrinsic fluorescence. Measurements were made employing 1-cm light path quartz cuvettes in a Perkin-Elmer LF-5B luminescence spectrometer, at room temperature. Stock solutions of purified Hsp80 were diluted into 20 mM Tris-HCl, pH 7.5, with the requisite amounts of the nucleotide to be tested, to a final protein concentration of 14 μg/mL. Individual samples were incubated for 5 min at room temperature and tryptophan fluorescence was followed at excitation and emission wavelengths of 295 and 339 nm, respectively. Fluorescence intensities were recorded following subtraction of the baseline readings obtained from the buffer. Changes in fluorescence are presented as relative values; corrections for inner filter effects were not made.

Analysis of Hsp70−Hsp80 Interaction on ATP−Agarose Columns. Sufficient amount of purified Hsp70 was loaded on to saturate a 0.5-mL ATP−agarose column preequilibrated with buffer B [10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 20 mM $MgCl_2$]. The column was next washed with 0.6 M NaCl to completely remove nonspecifically bound protein and reequilibrated with the starting buffer, prior to application of purified Hsp80. The latter was applied in parallel to an Hsp70-saturated and a buffer B-equilibrated ATP−agarose column. Heterooligomeric Hsp70−Hsp80 complexes were eluted with 5 mM ATP and the two proteins were detected by Western blotting with specific antibodies as described in the preceding sections.

Antibody Preparation and Protein Determination. The preparation of anti-Hsp80 IgG and that of anti-Hsp70 antiserum has been described previously (Roychowdhury et al., 1992a; Chakraborty et al., 1995). The protein content of different fractions was estimated employing the Bio-Rad micro assay using bovine serum albumin as a calibration standard (Bradford, 1976).

RESULTS

Purification of Hsp80. Hsp80 is present in nonshocked *N. crassa* mycelium in very low amounts but it is produced in massive quantities following heat-shock treatment for 1 h, such that the stress-induced protein is clearly visible in Coomassie blue-stained gels, while the other heat-shock proteins—with the exception of Hsp70—can be visualized only on labeling with ^{35}S-methionine (Kapoor & Lewis, 1987). On account of the prolific production of Hsp80 following heat shock and carbon starvation (Roychowdhury & Kapoor, 1988), we were able to develop a procedure for its purification, without the necessity for overexpression of the gene in a heterologous host.

The procedure used for purification of Hsp80, described in the Materials and Methods—a modification and improvement of our previously described method (Roychowdhury & Kapoor, 1990)—routinely yields approximately 450 μg of a nearly homogeneous preparation starting with 2 g of lyophilized heat-shocked mycelium. Fractionation on a Cibacron Blue−agarose column led to significant enrichment of the Hsp80 fraction, while fast protein liquid chromatography on a Mono Q column resulted in the final nearly homogeneous preparation (Figure 1, panel A). Aliquots from each of the successive purification steps, separated on SDS−polyacrylamide gels and electroblotted onto nitrocellulose membranes, were probed with specific polyclonal anti-Hsp80 IgG and anti-Hsp70 antisera to identify the protein bands corresponding to these two proteins (Figure 1, panels B C). These antibody preparations are specific for Hsp70 and Hsp80; no cross-reactivity was observed between these two or with any other heat-shock protein.

Subunit Structure of Hsp80 and the Effect of Nucleotides. The purified preparation of Hsp80 was employed to determine its quaternary structure in the native state. Glutaraldehyde (a 1,5-dialdehyde) is a bifunctional reagent believed to cross-link the ϵ-amino groups of lysine residues within a distance of 8 Å (Peters & Richards, 1977). Chemical cross-linking with glutaraldehyde followed by resolution on denaturing polyacrylamide gels revealed the presence of four prominent bands, corresponding to the unlinked monomeric

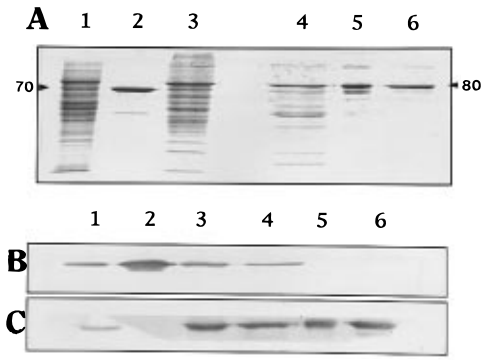

FIGURE 1: Analysis of protein profiles of fractions from different purification steps. (A) Equal amounts of protein (25 μg) from (1) crude extract of heat-shocked mycelium, (2) ATP−agarose column eluate, (3) DEAE-cellulose eluate, (4) Cibacron blue−agarose eluate fraction, (5) octyl-Sepharose fraction, and (6) Mono-Q eluate were analyzed by SDS−PAGE and Coomassie blue staining. Identical samples were electrotransferred to nitrocellulose and screened sequentially with anti-Hsp70 antibody (B) and, after removal of original probe, with anti-Hsp80 IgG (C).

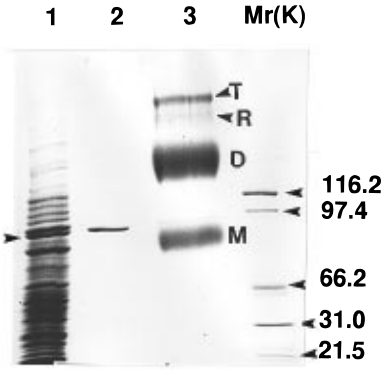

FIGURE 2: Coomassie blue-stained gel showing SDS−PAGE analysis of chemically cross-linked Hsp80. Lane 1, crude extract; lane 2, purified Hsp80; lane 3, purified Hsp80 sample cross-linked with glutaraldehyde. M, D, R, and T denote the non-cross-linked monomer and cross-linked dimeric, trimeric and tetrameric species, respectively. The molecular mass standards are shown in the extreme right-hand lane.

species and cross-linked dimers, trimers, and tetramers (Figure 2). These results suggest that the native state of Hsp80 is represented by an oligomeric structure composed of at least four subunits.

An approximate estimate of the molecular mass of the Hsp80 oligomer was obtained by gel filtration using a Superose-6 column (HR 10/30; Pharmacia), equilibrated against 20 mM Tris-HCl, pH 7.5 and calibrated with native molecular mass standards including thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The relative molecular mass of Hsp80 was shown to correspond approximately to 364 kDa (data not shown). The latter value is also consistent with a tetrameric structure, assuming the approximate molecular mass of 90 kDa for the protomer, as judged by resolution by SDS−polyacrylamide gel electrophoresis, along with the specified commercial molecular mass standards for denatured proteins.

While ATP is known to bind to the various members of the stress 70 family with high affinity, the Hsp90 stress family has been postulated to comprise ATP-independent molecular chaperones (Jacob & Buchner, 1994). Since there is some uncertainty as to whether ATP interacts with Hsp80, it was of interest to determine if incubation with ATP and

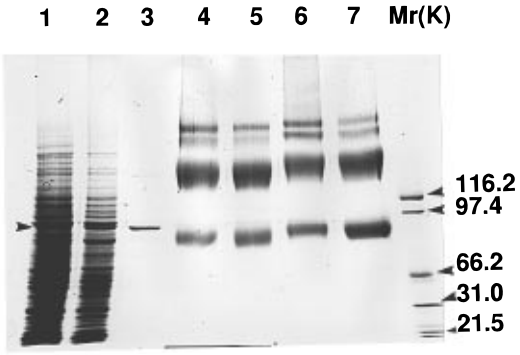

FIGURE 3: Effect of ATP on products of chemical cross-linking. Lanes 1 2, protein profiles of crude extracts of nonshocked cultures grown at 28 °C, for 16 h (1) and following heat shock treatment for 1 h at 48 °C (2); lane 3, untreated purified Hsp80; lane 4, Hsp80 sample cross-linked with glutaraldehyde without any additions; lanes 5 and 7, Hsp80 samples treated with 3 mM ATP prior to the cross-linking reaction; lane 6, cross-linking in the presence of 5 mM ATP. The arrowhead on the left indicates the position of the Hsp80 band in crude extracts of heat-shocked cells. The molecular mass markers are shown on the right.

other nucleotides had any effect on the subunit structure or conformation of Hsp80. Our earlier experiments with UV cross-linking of ^{32}P-labeled ATP had shown evidence of strong interaction with Hsp70 of *N. crassa*, while binding to Hsp80 was negligible (Roychowdhury & Kapoor, 1990). In view of the retention of Hsp80 by Cibacron Blue columns, it was reasonable to assume that it may contain structural motifs, capable of recognizing nucleotides.

To explore the effect of nucleotides on the subunit structure of Hsp80, the cross-linking reaction with glutaraldehyde was performed in the presence of varying concentrations of ATP. As shown in Figure 3, cross-linking in the presence of 3 and 5 mM ATP did not appear to alter, significantly, the overall balance between the population of cross-linked species relative to that of the monomer. Striking differences in the cross-linking pattern will not be observed unless major structural/conformational changes were propagated by interaction with ATP. However, conformational changes of a more subtle nature, not detectable by this technique, are not ruled out.

Estimates of the proportion of the cross-linked species—dimers, trimers, and tetramers—relative to the monomeric units, generated by glutaraldehyde cross-linking, demonstrate that the predominant species is the dimer in all instances. The comparative values of the cross-linked species versus non-cross-linked monomeric units produced by resolution of protein cross-linked in the presence and absence of ATP are presented in Figure 4. By averaging the values obtained from the densitometric analysis of protein profiles depicted in sets I−IV, the relative fractions of total protein in the monomeric species and cross-linked dimers are judged to be 27% and 38%, respectively, while the trimers and tetramers comprise 17% and 18% of the total. These results are consistent with a native tetrameric structure that is most likely to be a dimer-of-dimers.

In view of the binding of Hsp80 to Cibacron Blue−agarose, we decided to explore the effect of a few other nucleotides, including CTP, UTP, NAD(H), and NADP(H). These data are illustrated in Figure 5. Addition of 3 mM CTP did not have a noticeable effect on the cross-linking pattern, but in sharp contrast, in the presence of 5 mM CTP the relative level of un-cross-linked monomer diminished

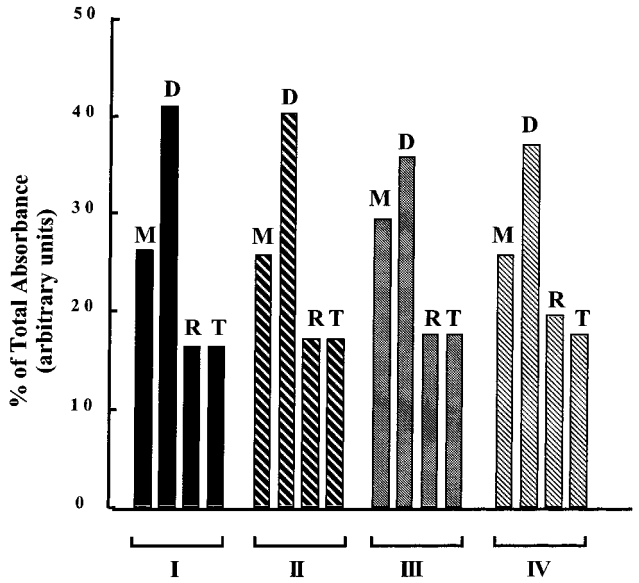

FIGURE 4: Relative levels of cross-linked species: densitometric analysis of the Coomassie blue stained gel in Figure 3. Results of computer-assisted densitometric scanning show the estimated relative amounts of the monomeric (M), dimeric (D), trimeric (R), and tetrameric (T) products, represented as percentage of total protein in the lane. I, control cross-linking profile, in the absence of any additions; II and IV, cross-linking in the presence of 3 mM ATP; III, cross-linking in the presence of 5 mM ATP.

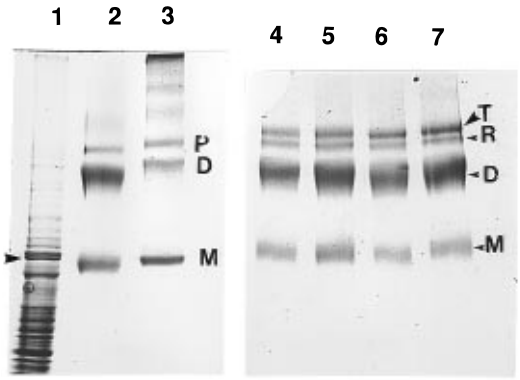

FIGURE 5: Effect of divalent cations and CTP on chemical cross-linking of Hsp80. Coomassie blue-stained, SDS−polyacrylamide gel showing heat-shocked cell extract (lane 1); purified Hsp80, cross-linked in the presence of 3 mM (lane 2) and 5 mM CTP (lane 3); lanes 4 and 5, cross-linked in the presence of 2.5 and 5.0 mM $MgCl_2$; and lanes 6 and 7, cross-linked in the presence of 2.5 and 5.0 mM $MnCl_2$. M, monomer; D, R, and T, cross-linked dimer, trimer, and tetramer.

(lane 3), compared to the control without any ligands (see Figure 3, lane 4) and, concomitantly, higher order oligomeric assemblies, greater than tetramers, were evident. Some of the higher aggregates, too large to penetrate the gel, can be seen at the top of the separating gel (Figure 5, lane 3). Oligomerization of Hsp80, for instance, in the presence of CTP—the tendency to form higher order homooligomeric assemblies *in vitro*—is suggestive of the possible occurrence of similar assemblies *in vivo*.

Next, the cross-linking reactions were performed in the presence of NAD and NADH. In the presence of 5 and 8 mM NADH, the monomer appeared in very low amounts while the dimeric, trimeric, and tetrameric species were not in evidence due to the failure of the higher order assemblies, incorporating a majority of subunits, to enter the gel (not shown). The presence of 2 and 5 mM $MgCl_2$ and $MnCl_2$

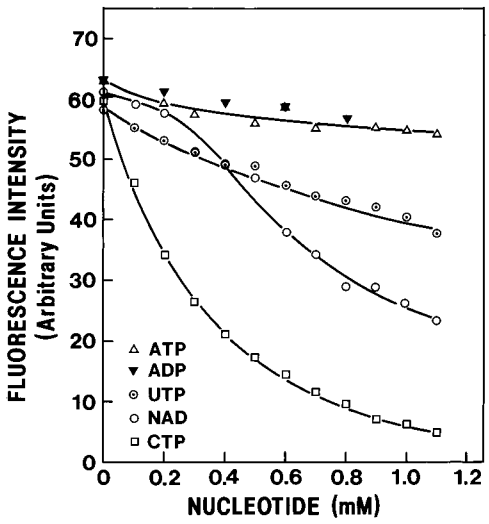

FIGURE 6: Effect of ligands on the intrinsic fluorescence of Hsp80. Tryptophan fluorescence of Hsp80 (140 μg/mL) upon addition of indicated concentrations of nucleotides was measured using an excitation wavelength of 295 nm and emission at 339 nm.

during the cross-linking reaction did not have a pronounced effect on the relative proportion of the unlinked versus the various cross-linked species (Figure 5, lanes 4−7). However, when 2.5 or 5 mM $MgCl_2$ was employed in combination with 3.5 mM ATP, the majority of the protein was recovered in the form of higher order aggregates that failed to penetrate the gel, as was the case when a combination of 3.5 mM each NAD and ATP was employed (not shown).

Fluorescence Studies of Interaction of Hsp80 with Nucleotides. The preceding experiments demonstrated that Hsp80 in its native state is an oligomer, capable of interacting with nucleotide triphosphates, such as ATP and CTP, and dinucleotides, such as NAD(P) and NAD(P)H. To verify the interaction between Hsp80 and nucleotides, intrinsic tryptophan fluorescence of Hsp80 was followed as a function of interaction with the nucleotides. Binding of the nucleotide ligands was assessed by monitoring quenching of tryptophan fluorescence by following the emission at 339 nm, upon excitation at 295 nm, by inclusion of 1-μL increments of stock solutions of a given ligand. Addition of ATP and ADP did not result in a significant change in tryptophan fluorescence (Figure 6). In striking contrast, NAD and CTP led to pronounced quenching, the effect of the latter being the most dramatic. These data represent relative values as corrections were not made for inner filter effects, originating from the absorbance of ligands at the exciting wavelength; correction for the contribution of absorbance at 295 nm, by the nucleotide used, would yield qualitatively similar fluorescence profiles. Considered together with the results of cross-linking experiments, the fluorescence data demonstrate that NAD and CTP do indeed bind to Hsp80, thereby propagating conformational changes in the protein leading to alterations in its quaternary structure.

Interaction of Hsp80 with Hsp70. As both Hsp80 and Hsp70, the most abundant stress-induced Hsps of *N. crassa*, are cytosolic proteins, it was of interest to determine if these two molecular chaperones interacted with each other. Molecular chaperones are known to frequently function in concert with other effectors or cochaperones. Furthermore, there may be a functional overlap in the action of major molecular chaperones. The results of our previous experi-

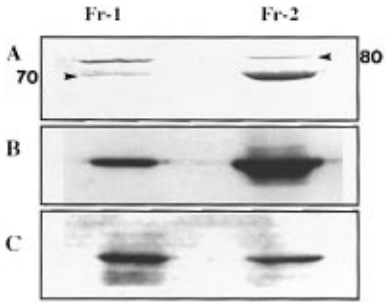

FIGURE 7: Retention of Hsp80 by Hsp80-saturated ATP-agarose. (A) SDS−PAGE analysis of [Hsp70−Hsp80] complex, eluted by using two consecutive 500-μL aliquots of 5 mM ATP (Fr = fraction). Western blots of ATP-eluted proteins using anti-Hsp70 (B) and anti-Hsp80 (C) antibodies are shown. The arrowheads in panel A indicate Hsp70 and Hsp80.

ments demonstrating that inactivation of either Hsp70 or Hsp80 alone, by means of the RIP (*r*epeat-*i*nduced *p*oint mutations) process, resulted in slower-than-normal growth but did not altogether eliminate the capacity of the cells to develop thermotolerance (Chakraborty et al., 1995; Vijayaraghavan & Kapoor, 1996) support the hypothesis of a functional overlap.

To assess the interaction between Hsp70 and Hsp80, purified Hsp70 was immobilized on ATP−agarose by loading 15 μg on a 0.5-mL column. The column was washed thoroughly with the starting buffer to ensure complete removal of unbound protein, following which an equivalent quantity of purified Hsp80 was applied to the same column. The effluent was tested for unbound Hsp80 by Western blot analysis of the wash fractions. While ATP−agarose failed to bind Hsp80 by itself, upon prior saturation with Hsp70 the same matrix retained Hsp80 effectively, showing that immobilized Hsp70 was responsible for subsequent retention of Hsp80. Upon passage of 5 mM ATP through the column—which dissociates Hsp70 completely from the matrix—the eluted fractions were subjected to SDS−PAGE followed by Western blot analysis of the same membrane, in succession, with Hsp70- and Hsp80-specific antibodies. Western blot analysis of the eluted protein revealed the presence of Hsp70 and Hsp80 together; no cross-reactivity was observed between them (Figure 7). As illustrated in Figure 7, both Hsp70- and Hsp80-specific antibodies identified the corresponding protein in the same factions (Fr-1 and Fr-2). The retention of Hsp80 by ATP−agarose, saturated with Hsp70, and its coelution with Hsp70 suggested the formation of a complex between these two proteins. Support for an interaction between Hsp70 and Hsp80 was also furnished by immunoprecipitation, where the specific antibodies raised against Hsp70 and Hsp80, were observed to precipitate both proteins (not shown).

Further evidence for a direct interaction between Hsp70 and Hsp80 was obtained by treatment of Hsp70, Hsp80, and a 1:1 mixture of the two with glutaraldehyde as outlined under Materials and Methods, followed by resolution of the cross-linked material by SDS−PAGE (Figure 8, panel A). The photograph of the Coomassie blue-stained gel shows the bands corresponding to Hsp70 and Hsp80 in the crude extract and the homooligomeric products of intraprotein cross-linking of Hsp80 (panel A, lane 4). Upon transfer to a nitrocellulose membrane, the protein bands in a similar gel were screened by Western blot analysis, which revealed cross-linked homooligomers of Hsp70 on probing with anti-

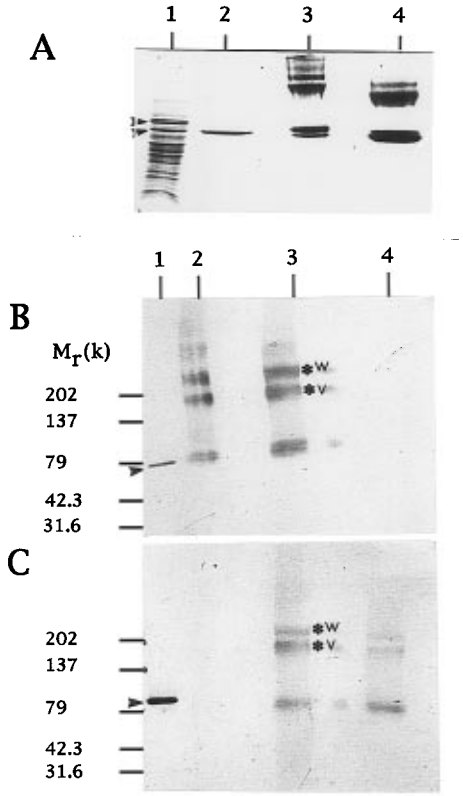

FIGURE 8: Analysis of chemically cross-linked, purified Hsp70 and Hsp80 individually and in combination. (A) Separation by SDS−PAGE and Coomassie blue staining of heat-shocked cell extract (1), chemically cross-linked Hsp70, (2), Hsp70/Hsp80 (3), and Hsp80 (4). The same Western blot of cross-linked proteins was sequentially probed using anti-Hsp70 (B) and anti-Hsp80 (C) antibodies. The position of Hsp70 and Hsp80 in lane 1 of panels A, B and C is indicated by arrowheads. Heterooligomeric complexes containing both Hsp70 and Hsp80 are indicated by asterisks.

Hsp70 antiserum (Figure 8, panel B, lane 2), although only un-cross-linked monomers were visible in the stained gel (Figure 8, panel A, lane 2), showing that a small fraction of Hsp70 existed in the dimeric and higher oligomeric forms. Homooligomers of Hsp80 were visualized on reaction with anti-Hsp80 IgG (panel C, lane 4). It is noteworthy that the reaction of Hsp80 antibodies was stronger with the Hsp80 band in the crude extract (panel C, lane 1) as opposed to that with the various cross-linked species (panel C, lane 4). This difference is attributable to structural change in the Hsp80 subunits, as a consequence of inter- and intrasubunit cross-linking, resulting in a direct or indirect masking of epitopes, normally available in the non-cross-linked form. In addition, covalently cross-linked heterooligomeric assemblies, containing Hsp70 and Hsp80 polypeptides, were revealed by both anti-Hsp70 and anti-Hsp80 antibodies (indicated by asterisks in Figure 8, panels B and C), thus confirming a direct interaction between these two proteins. A comparison of the polypeptide bands displayed in lane 3 of panels B and C suggests that the relative contribution of the Hsp80 and Hsp70 subunits is apparently equal in the oligomeric band *v, while the composition of band *w probably represents a ratio of 2:1 of Hsp70 and Hsp80. As electrotransfer of larger, higher order complexes is relatively inefficient, the number of Hsp70 and Hsp80 protomers participating in the formation of the nondenatured heterocomplexes will be difficult to assess on the basis of these experiments.

DISCUSSION

The results of chemical cross-linking experiments presented in the foregoing suggest that Hsp80 of *Neurospora* is a homooligomer, existing as a tetramer or as a higher order assembly in the native state. These results are supported by gel-filtration studies that yielded an estimate of approximately 364 000 Da for the relative molecular mass of the native protein. Interestingly, our previous gel-filtration studies conducted in the presence of 100 mM Tris-HCl (Roychowdhury & Kapoor, 1990) had yielded a M_r value approximately 610 000 for the native Hsp80. The current estimates are based on gel filtration in 20 mM Tris-HCl. The difference in the two estimates may be attributable to the ionic strength of the buffer influencing the formation of a higher order oligomeric structure, based on weak intersubunit interactions. An apparent molecular mass value of 360 kDa, consistent with a tetramer, determined by gel filtration has also been reported recently for the yeast Hsp90 (Jacob et al., 1995). In contrast, investigations with homologues from other organisms have provided evidence indicating that Hsp90 exists as a dimer (Minami et al., 1991; Welch & Feramisco, 1982; Spence & Georgopoulos, 1989).

Calculations of apparent molecular mass from gel-filtration profiles are based on the assumption of a globular shape for the protein, hence potentially leading to erroneous values for nonglobular proteins. For instance, by measurements of sedimentation constant and Stokes' radius the native state of purified Hsp90 from chicken liver was inferred to be an elongated dimer of ~160 000, characterized by a high frictional ratio, while gel-filtration studies had suggested a molecular mass close to 500 000 (Iannotti et al. 1988). High frictional ratios are indicative of an asymmetrical, rather than globular, structure. Hydrodynamic studies led to a similar conclusion with respect to the structure of a homologous protein from rabbit reticulocytes (Rose et al. 1987).

Our studies with chemical cross-linking have led to the recognition of some novel structural features of Hsp80 of *N. crassa*, outlined in the following. The relative distribution of the cross-linked species suggests that the quaternary structure of the *N. crassa* Hsp80 tetramer may comprise a dimer of dimers. The reasoning for this conclusion is as follows. If the contacts between all of the individual protomers in the quaternary structure were assumed to be identical, on theoretical grounds a single cross-link will be expected to occur at a higher frequency than two cross-links per tetramer, while the frequency of three cross-links to generate a cross-linked tetramer would be correspondingly lower. The expected relative distribution of cross-linked species and un-cross-linked monomers would be monomer > dimer > trimer > tetramer. However, in all of the cross-linking reactions the cross-linked dimer was recovered in the highest relative yield. The trimer was observed at a lower level relative to the dimeric and the monomeric species but virtually equal to that of the tetramer, indicating that the intersubunit cross-linking was not a random event and that the dimeric units comprised the most abundant and favored species. This can be explained in terms of a dimer-of-dimers structural construct for the native protein: if cross-links between one type of like binding pairs (isologous contacts), were to occur more readily than another type of isologous (or heterologous) contact sites, the observed relative yields of the various cross-linked species would be expected.

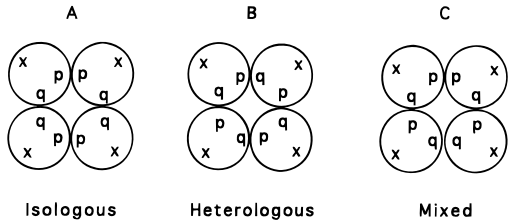

FIGURE 9: Diagrammatic representation of a planar tetramer with hypothetical intersubunit bonding domains. Isologous contacts are shown by pp and qq bonding pairs, and pq contacts denote heterologous interactions. Unlinked, open, bonding domains are indicated by x.

The following hypothetical model for the quaternary structure of Hsp80, based on our cross-linking data, can be considered (Figure 9). Although a tetrahedral arrangement of protomers is possible, it will be assumed for the purpose of this discussion that a planar arrangement is more plausible. According to the classical theoretical treatment presented by Cornish-Bowden and Koshland (1971), the possible planar arrangement of subunits in a tetramer with identical subunits can consist of exclusively heterologous or isologous, as well as mixed interprotomer bonding domains, where pp- and qq-type bonding pairs are defined as isologous and pq pairs are heterologous. In Figure 9, a diagram of a planar tetramer with these three arrangements is shown, and for the sake of simplicity, only one mixed arrangement is depicted. Bonding domains participating in intersubunit interactions in a tetramer are denoted by the symbols p and q, while x represents the putative open or free contact domains. The assumption of open interprotomer bonding domains is reasonable in view of our observation of higher order oligomers (i.e., with CTP). A structure with all heterologous interprotomer contacts (B) is not compatible with our experimental data, which show the cross-linked dimer to be the predominant species. On the other hand, structures with all isologous (A) or two isologous and two heterologous bonding domains (B) will be able to generate cross-linking patterns described for Hsp80, if the assumption were made that, for instance, pp bonding pairs were prone to form cross-links more readily than qq pairs or *vice versa*; likewise, for mixed domains, if the assumption were made that isologous interactions (pp and qq) were stronger than heterologous (pq) ones. However, arrangements involving isologous and heterologous interactions, as in C, are likely to be implausible. Therefore, model A remains the most likely arrangement for the native state of Hsp80. This arrangement is also compatible with formation of higher order homooligomers, resulting from ligand-induced conformational changes, as well as with the formation of heterooligomeric assemblages with Hsp70, and possibly, with other proteins.

That some of the nucleotides interact with Hsp80 and propagate conformational changes is suggested by the quenching of tryptophan fluorescence as well as our cross-linking data. In one respect, the yeast Hsp90 exhibits a similar behavior in that ATP was reported not to exert any influence on the cross-linking pattern but differs in response to divalent cations: millimolar amounts of $MgCl_2$ and $MnCl_2$ enhanced the formation of cross-linked dimers and tetramers (Jacob et al., 1995). In contrast, these cations were without any noticeable effect on the *N. crassa* Hsp80.

The binding of pyrimidine nucleotides and dinucleotides has a more pronounced effect on the conformation of the *N.*

crassa Hsp80 than that of purine nucleotides. Extensive studies have been conducted with the eukaryotic Hsp70 and its prokaryotic analogue DnaK, and it is known that the Hsp70 family members are characterized by a high-affinity ATP binding site in the N-terminal domain and ATPase activity (Flaherty et al., 1990). The binding of Mg^{2+}-ATP, rather than its hydrolysis, has been found to be essential for its chaperoning interaction with peptide ligands (Schmid et al., 1994; Palleros et al., 1993). The ATPase activity of DnaK is stimulated by binding of the substrate and a monovalent cation, such as K^+, is required for this step, as well as for stabilization of the [DnaK−ADP] complex and release of the substrate (Feifel et al., 1996).

While the role of ATP vis-a-vis the chaperoning behavior of the Hsp70 family is well-established, direct involvement of ATP with Hsp90 is still a matter of debate. Evidence showing the ability of Hsp90 to bind and hydrolyze ATP was presented earlier (Nadeau et al., 1992; Csermely et al., 1993). More recently, Jacob et al. (1996) conducted a thorough reevaluation of this question using meticulously prepared reagents along with highly purified and nucleotide-free yeast and bovine Hsp90. Their detailed study on the effect of ATP on intrinsic fluorescence of Hsp90, experiments with cross-linking to 8-azido-ATP and binding of etheno-ADP (a fluorescent derivative of ADP), together with assays of ATPase activity, provided convincing evidence for a lack of binding of ATP or ATP hydrolysis by Hsp90. However, results of another study, based on data derived from purified human hsp90β (Sullivan et al. 1997) support its binding to ATP with a relatively low affinity, resulting in conformational change(s) leading to increased complex formation with p23. These contradictory reports on the response of Hsp90 to nucleotides—particularly ATP and ADP—may be ascribable to individual differences in structure/properties of this protein in different organisms.

As stated in the foregoing, our studies with *Neurospora* Hsp80 show virtually no quenching of intrinsic tryptophan fluorescence by ATP or ADP. The effect of pyrimidine nucleotides and that of the dinucleotides constitutes a novel observation for the *N. crassa* Hsp80(90). The formation of higher order assemblies in the presence of specific nucleotide ligands, such as CTP and NAD, may be a reflection of specialization in the chaperoning function performed by Hsp80, in response to intracellular physiological conditions or vis-a-vis its substrate specificity. The direct interaction of purified Hsp70 and Hsp80 was clearly witnessed by retention of Hsp80 on ATP-agarose columns. While this matrix binds Hsp70 with high affinity, no binding of Hsp80, by itself, was observed. On the other hand, if the matrix was first saturated with Hsp70, retention of Hsp80 was evident, showing its binding to Hsp70 previously immobilized on ATP−agarose. Elution of the column with 5 mM ATP resulted in the coelution of Hsp70 and Hsp80 in the same fractions. These results were corroborated by our cross-linking experiments where heterooligomeric complexes containing Hsp70 and Hsp80 were revealed by immunoblot analysis of products of cross-linking, electrophoretically resolved in denaturing polyacrylamide gels. The heterooligomeric Hsp70−Hsp80 complexes shown in Figure 8 appear to be in the size range commensurate with heterodimers or trimers/tetramers but the number of Hsp70 and Hsp80 subunits involved in these assemblies, prior to denaturation, is difficult to determine with accuracy. Our unpublished experiments (to be reported elsewhere) using the enzyme-linked immunosorbent assay (ELISA) system, have provided additional evidence confirming the formation of heterooligomeric complexes containing Hsp80 and Hsp70. Furthermore, the nucleotides, particularly CTP and NAD, were observed to exert a pronounced stimulatory effect on the formation of the [Hsp70−Hsp80] complex, as a consequence of their binding to Hsp80 (P. M. Ouimet and M. Kapoor; manuscript in preparation).

In mammalian hormone receptor systems Hsp90 and Hsp70 are thought to act synergistically. Using Hsp70-depleted rabbit reticulocyte lysate containing Hsp90, Hutchinson et al. (1994) showed that Hsp70 was necessary for complexation of the glucocorticoid receptor *in vitro* with Hsp90, presumably on account of its unfolding ability. The formation of heterooligomeric assemblies containing hormone receptors, mammalian Hsp70 and Hsp90 (equivalent of *N. crassa* Hsp80) and other system-specific components, such as the cyclophilin FKBP52/Hsp56, cyt40, and Mas70, provides a molecular basis for the cooperative chaperoning action of these two Hsps (Owens-Grillo et al., 1996). However, in the mammalian systems there is no evidence for a direct interaction of Hsp70 and Hsp90.

Compelling evidence for a concerted/sequential action of molecular chaperones has emerged from various studies, and evidently multiple folding pathways may operate in the same cell (Hartl, 1996). In *E. coli*, even in the absence of external stress, the contribution of both DnaK/DnaJ/GrpE and GroEL/GroES chaperoning systems appears to be necessary for protection of diverse nascent polypeptides from aggregation (Gragerov et al., 1992). Moreover, the functional cooperation of Hsp70 and the chaperonin families has also been confirmed *in vitro* (Langer et al., 1992). How the *Neurospora* Hsp70/Hsp90 complexes function in the chaperoning process *in vivo* and whether these complexes may entail recruitment of additional cofactors, such as equivalents of prokaryotic DnaJ and yeast YDJ1, to generate a macromolecular folding apparatus with optimal activity (or substrate specificity) are pressing questions for future studies. Experiments are currently in progress to evaluate the chaperoning ability of Hsp70 and Hsp90 individually, as well as that of the complex containing these two proteins.

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