Protein Science (1999), *8*:905–912. Cambridge University Press. Printed in the USA.

Nucleolar protein B23 has molecular chaperone activities

ATTILA SZEBENI AND MARK O.J. OLSON

Department of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505

(RECEIVED November 2, 1998; ACCEPTED December 29, 1998)

Abstract

Protein B23 is an abundant, multifunctional nucleolar phosphoprotein whose activities are proposed to play a role in ribosome assembly. Szebeni et al. (1997) showed stimulation of nuclear import in vitro by protein B23 and suggested that this effect was due to a molecular chaperone-like activity. Protein B23 was tested for chaperone activities using several protein substrates. The temperature-dependent and -independent aggregation of the HIV-1 Rev protein was measured using a zero angle light scattering (turbidity) assay. Protein B23 inhibited the aggregation of the Rev protein, with the amount of inhibition proportional to the concentration of B23 added. This activity was saturable with nearly complete inhibition when the molar ratio of B23:Rev was slightly above one. Protein B23 also protected liver alcohol dehydrogenase (LADH), carboxypeptidase A, citrate synthase, and rhodanese from aggregation during thermal denaturation and preserved the enzyme activity of LADH under these conditions. In addition, protein B23 was able to promote the restoration of activity of LADH previously denatured with guanidine-HCl. Protein B23 preferentially bound denatured substrates and exposed hydrophobic regions when complexed with denatured proteins. Thus, by several criteria, protein B23 behaves like a molecular chaperone; these activities may be related to its role in ribosome biogenesis.

Keywords: HIV-1 Rev protein; molecular chaperone; nucleolar protein B23; protein denaturation; protein folding

The nucleolus is a compact subnuclear structure specializing in the assembly of ribosomal subunits in eukaryotes (Busch & Smetana, 1970; Hadjiolov, 1984). This is a multistep process beginning with the transcription of preribosomal RNA (pre-rRNA), followed by processing of the pre-rRNA to 18S, 5.8S, and 28S ribosomal RNAs, addition of 5S rRNA and attachment of ribosomal proteins (Warner, 1990). The assembly process requires numerous nonribosomal proteins (Olson, 1990). Some of these proteins are associated with the RNA polymerase I transcriptional apparatus (Moss & Stefanovsky, 1995) while others are involved with the processing of the 47S preribosomal RNA transcript (Eichler & Craig, 1994). However, the functions of many other nucleolar nonribosomal proteins are poorly understood.

The concentration of macromolecules in living cells is extremely high, with estimates as high as 200–400 mg/mL (Zimmerman & Minton, 1993; Ellis, 1997). The density of the nucleolus suggests that it also contains a high concentration of proteins, especially during periods of active ribosome assembly. A major consequence of this "macromolecular crowding" could be the aggregation and immobilization of proteins in the process of being incorporated into ribosomes. The tendency for proteins to aggregate is generally relieved in many biological systems by the action of molecular chaperones (Ellis, 1997). Since ribosome assembly requires precise coordination of events involving a large number of proteins in a crowded environment, one can imagine the need for chaperone-like molecules to prevent aggregation in the nucleolus.

One possible candidate for a nucleolar chaperone is an abundant nonribosomal protein called B23, which is also known as NO38 (Schmidt-Zachmann et al., 1987), numatrin (Feuerstein & Mond, 1987), or nucleophosmin/NPM (Chan et al., 1989). This was suggested by recent studies showing effects of protein B23 on nuclear import. Protein B23 was previously shown to bind peptides containing nuclear localization signals (NLSs), based on the SV40 T-antigen NLS (Goldfarb, 1988). The HIV-1 Rev protein also binds strongly to protein B23 (Fankhauser et al., 1991) and accumulates in the nucleolus. We found that the affinity of the Rev NLS for B23 is about 10-fold higher than that of the T-antigen NLS (Szebeni et al., 1995). The latter finding, coupled with the nucleocytoplasmic shuttling behavior of B23 (Borer et al., 1989), led to an examination of the effect of protein B23 on nuclear import of the Rev protein (Szebeni et al., 1997). It was found that protein B23 stimulates the import of Rev into the nucleus with maximal stimulation occurring at a 1:1 molar ratio of the two proteins. Since the Rev protein has a marked tendency to aggregate under normal physiological conditions (Wingfield et al., 1991), a possible interpretation of these results is that protein B23 acts as a molecular chaperone to maintain the solubility of Rev and improve its mobility during import into and throughout the nucleus. This same activity could

Reprint requests to: Mark O.J. Olson, Department of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505; e-mail: molson@biochem.umsmed.edu.

Abbreviations: ANS, 1-anilino-8-sulfonate; BSA, bovine serum albumin; CPA, carboxypeptidase A; CS, citrate synthase; DTT, dithiothreitol; LADH, liver alcohol dehydrogenase; NLS, nuclear localization signal; pre-rRNA, preribosomal RNA; RNP, ribonucleoprotein.

be utilized to prevent the aggregation of other proteins in the nucleolus and aid in the ribosome assembly process.

The characteristics of protein B23 described above suggested that it has molecular chaperone activity. To test this possibility, we initially developed an assay to measure its effects on the temperature-dependent aggregation of the HIV-1 Rev protein. It was found that protein B23 inhibits Rev protein aggregation. This finding raised the possibility that protein B23 also has more general chaperone activity toward a variety of substrates. Assays for activities typical of many chaperones showed that it prevents aggregation and preserves enzyme activities during thermal denaturation of several different proteins, promotes the renaturation of previously denatured proteins, preferentially binds denatured proteins, and exposes hydrophobic regions during interaction with other proteins. These studies support the idea that protein B23 is a molecular chaperone.

Results

Protein B23 inhibits aggregation of the HIV-1 Rev protein

The Rev protein undergoes reversible temperature-dependent or -independent aggregation, depending on the conditions of ionic strength and sulfate concentration (Wingfield et al., 1991). To quantify the temperature-dependent aggregation, we used a zero angle light scattering (turbidity) assay. The absorbance was monitored at 360 nm as the temperature rose from 0 to 30 °C in a spectrophotometer cuvette after adding ice-cold Rev solution. Figure 1A shows that there was a relatively rapid aggregation phase over a period of 40–50 min followed by a gradual approach toward maximum turbidity within about 120 min. If the polymerized Rev protein was cooled again to 0 °C, there was a slow disappearance of turbidity after approximately one day (data not shown). In contrast, if an equimolar concentration of protein B23 was added to the Rev protein solution, the rapid phase of the aggregation was prevented, although there was a slow increase in absorbance at 360 nm (Fig. 1A). When an equimolar concentration of BSA was used as a control, there was no effect on Rev protein aggregation. Protein B23 exists as two isoforms (Chang & Olson, 1990) with B23.2 containing 35 fewer amino acid residues at the C-terminal end compared to B23.1. To determine whether there were differences between the two isoforms in preventing Rev aggregation, we did similar experiments using protein B23.2 instead of B23.1. Figure 1A shows that the effects of the two isoforms on Rev aggregation were nearly identical, suggesting that the C-terminal end of B23.1 does not play a role in this activity.

To determine the molar ratio for the maximum inhibition of Rev aggregation, we repeated the assay as shown in Figure 1A using various concentrations of protein B23.1. A separate curve was generated for each concentration of protein B23 and the percent inhibition at the 20 min time point was plotted vs. the molar ratio of protein B23 to Rev (Fig. 1B). The inhibition of aggregation increased as the B23 concentration was raised, resulting in a hyperbolic curve. A plateau of inhibition of aggregation was seen when the molar ratio of B23:Rev was above one. This result suggested that under these conditions the Rev protein forms a relatively stable complex with protein B23 and that the maximal effect is achieved at a 1:1 stoichiometry between the two proteins.

At low ionic strength the Rev protein undergoes a slow polymerization in a temperature-independent manner with formation of

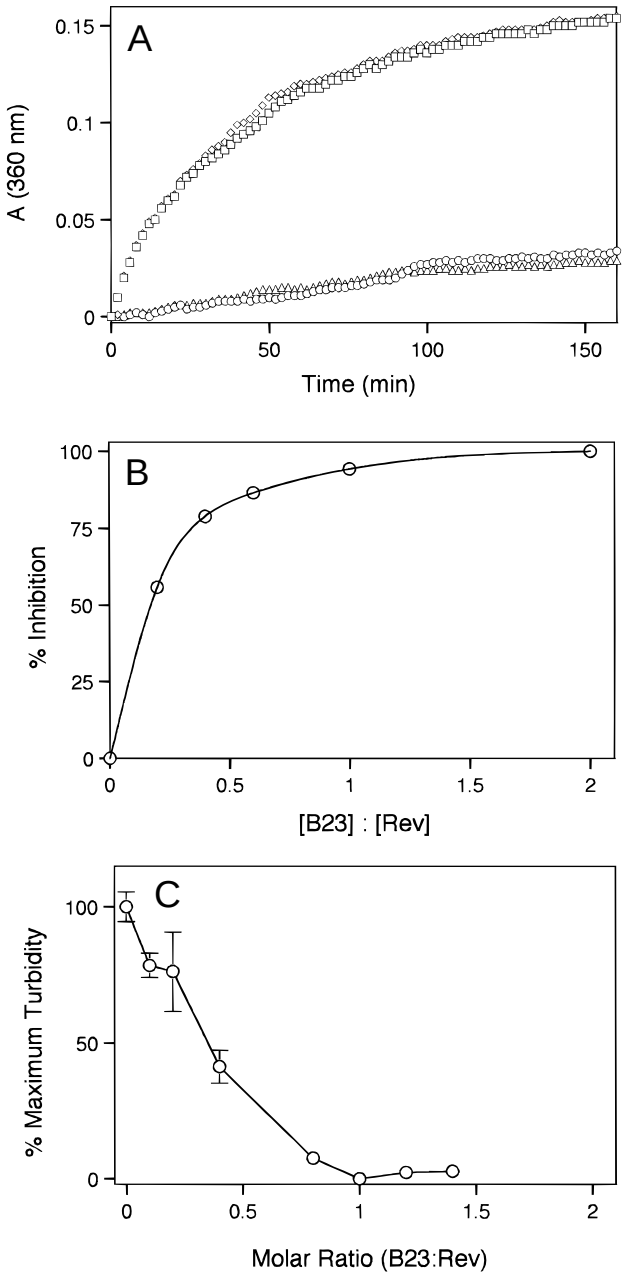

Fig. 1. Chaperone activity of protein B23 against the HIV-1 Rev protein. **A:** Effect of protein B23 on temperature-dependent aggregation of the HIV-1 Rev protein. Solutions of Rev protein (0.1 mg/mL in 50 mM phosphate buffer, pH 7.2, containing 150 mM NaCl, 1 mM DTT) without (□) or with equimolar amounts of added B23 (○, B23.1; △, B23.2) were allowed to warm from 4 to 28 °C in a semi-micro cuvette. The Rev aggregation assay was also done in the presence of an equimolar concentration of BSA (◇). The turbidity was monitored at 360 nm as described in Materials and methods. **B:** Protein B23 concentration dependence of inhibition of Rev aggregation. Turbidity assays were performed as in panel A with increasing concentrations of protein B23.1. The percent inhibition for each concentration of B23.1 was taken at the 20 min time point. The data are shown as means of three determinations. **C:** Effect of protein B23 on temperature-independent aggregation of the Rev protein. Aliquots of Rev solution (0.5 mg/mL in 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, 200 mM KCl, and 1 mM DTT) were dialyzed against 20 mM phosphate buffer (pH 7.0) containing 1 mM DTT at 4 °C. The samples included increasing amounts of protein B23.1 as indicated by the ratios of Rev:B23. The turbidity was measured at 360 nm after overnight dialysis. Data are expressed as the percentage of the maximum turbidity (Rev without added protein B23). The data are shown as means of two determinations.

insoluble fibrous material (Wingfield et al., 1991). To test whether protein B23 also inhibited the temperature-independent polymerization of Rev, aliquots of Rev in high ionic strength buffer (approximately 400 mM) were dialyzed in micro dialyzer cassettes against low ionic strength buffer (20 mM) at 4 °C. The samples included various concentrations of protein B23.1. The turbidity was measured as above after overnight dialysis. The samples containing Rev alone showed the greatest level of turbidity, whereas addition of increasing concentrations of protein B23 resulted in decreasing turbidity (Fig. 1C). As with the temperature-dependent aggregation described above, the solutions were essentially clear when the B23:Rev ratio was greater than one. The Rev protein also has a tendency to polymerize and form filaments over a period of several days at 0–4 °C, when its concentration is 1–2 mg/mL and under moderate ionic strength conditions (Wingfield et al., 1991). Addition of protein B23 also completely prevented this time-dependent Rev polymerization (data not shown). Thus, the ability of protein B23 to inhibit Rev aggregation can be seen under several different conditions of ionic strengths and temperature.

Protein B23 protects several proteins against thermal denaturation

The above studies provided evidence that protein B23 has chaperone-like activity toward a protein with which it is known to interact. To determine whether the protein has general chaperone activity, we studied the effect of protein B23 on the thermal denaturation of four enzymes that have previously been used as substrates for chaperones: liver alcohol dehydrogenase (LADH), carboxypeptidase A (CPA), citrate synthase (CS), and rhodanese. Light scattering measurements showed that at a concentration of 100 μg/mL LADH aggregated when the temperature was increased from 0 to 37 °C reaching maximum turbidity in about 40 min (Fig. 2A). However, when a 1:1 molar ratio of protein B23 was added to the incubation medium containing LADH, the aggregation was nearly completely prevented. The protein B23.2 isoform showed the same anti-aggregation effect on LADH as did B23.1 (not shown), again suggesting that the C-terminal end of protein B23.1 does not play role in the chaperone activity. In control experiments the presence of excess bovine serum albumin did not prevent the increases in light scattering (data not shown).

Similar analyses were performed on the other three enzymes; the results were generally very similar to those obtained with LADH when stoichiometric amounts of protein B23.1 was present during denaturation. However, with CPA (Fig. 2B) and CS (Fig. 2C), only partial inhibition of aggregation was achieved at the 1:1 stoichiometry. On the other hand, complete protection against aggregation was obtained under these conditions with rhodanese. In control experiments, the presence of an equimolar concentration of BSA did not alter the magnitude of aggregation (data not shown).

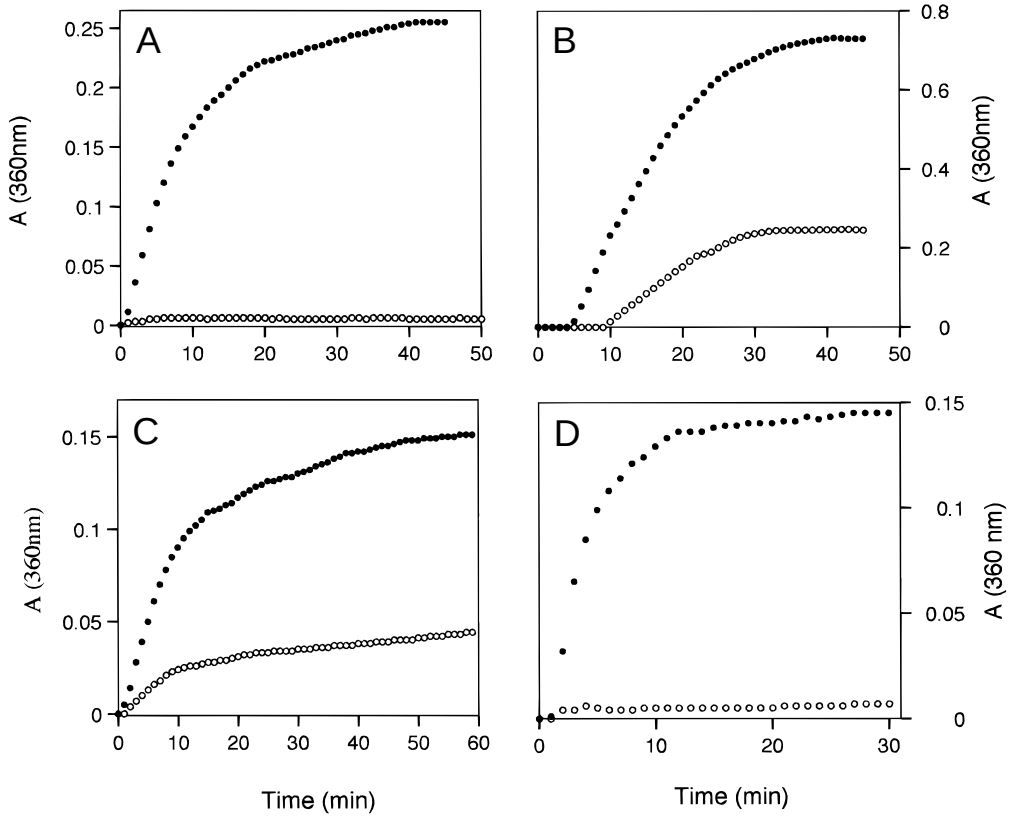

Fig. 2. Effect of protein B23 on the thermal denaturation of various substrate proteins. The proteins were subjected to thermal denaturation at the temperatures indicated below in the absence (●) or presence (○) of an equimolar concentration of protein B23.1 as described in Materials and methods. The turbidity was monitored at 360 nm. **A:** Horse liver alcohol dehydrogenase (37 °C). **B:** Bovine pancreatic carboxypeptidase A (48 °C). **C:** Citrate synthase (43 °C). **D:** Rhodanese (65 °C). All data are expressed as means of three experiments.

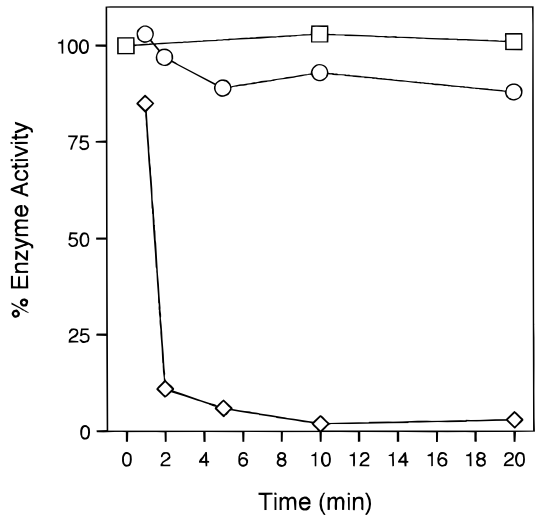

Fig. 3. Effect of protein B23 on the enzyme activity of liver alcohol dehydrogenase during thermal denaturation. LADH was incubated at 50 °C in the presence (○) or absence (◇) of protein B23.1 at a molar ratio of 1:1 (B23:LADH). A LADH control without added protein B23.1 was kept at 22 °C (□). The enzyme activities were assayed as described in Materials and methods. All data represent the means of three experiments.

Enzyme activity is a more sensitive measure of the native or denatured state of a protein than the aggregation assay. Figure 3 shows the effect of protein B23 on the time course of thermal denaturation of LADH as measured by enzyme activity. LADH was incubated at 50 °C in the absence or presence of the protein B23.1 isoform at a molar ratio of 1:1 (B23:LADH). During heating, LADH lost enzyme activity very rapidly, i.e., within 2 min. However, when protein B23 was present the enzyme retained nearly complete activity. In the control experiments not shown, it was found that the addition of B23 to an untreated solution of LADH did not influence the specific activity of the enzyme, nor did the presence of BSA protect the activity of LADH under the conditions used. Thus, protein B23 preserves the activity of LADH under high temperature conditions.

Protein B23 promotes the renaturation of chemically denatured proteins

Many molecular chaperones not only protect proteins against the effects of elevated temperatures, but they are also capable of promoting the refolding of denatured proteins. Two substrates, LADH and rhodanese, were used to test for this possible activity in protein B23. LADH was denatured in 6 M guanidine hydrochloride and diluted 100-fold as described in Materials and methods. The catalytic activity of the enzyme was measured after refolding in the absence or presence of protein B23 at a molar ratio of 1:1 (B23:LADH). Figure 4A shows the time course of reactivation of LADH by protein B23. The enzyme was completely inactive after denaturation and dilution, although there was a very slow restoration of the activity after several hours at room temperature. However, when B23 was present, 76% of the original activity was regained after 3 h, compared to only about 20% in the untreated sample. In control experiments it was found that the addition of B23 to a native solution of LADH did not influence the specific activity of the enzyme (data not shown). Furthermore, addition of BSA to the renaturing solution did not accelerate the recovery of enzyme activity (data not shown).

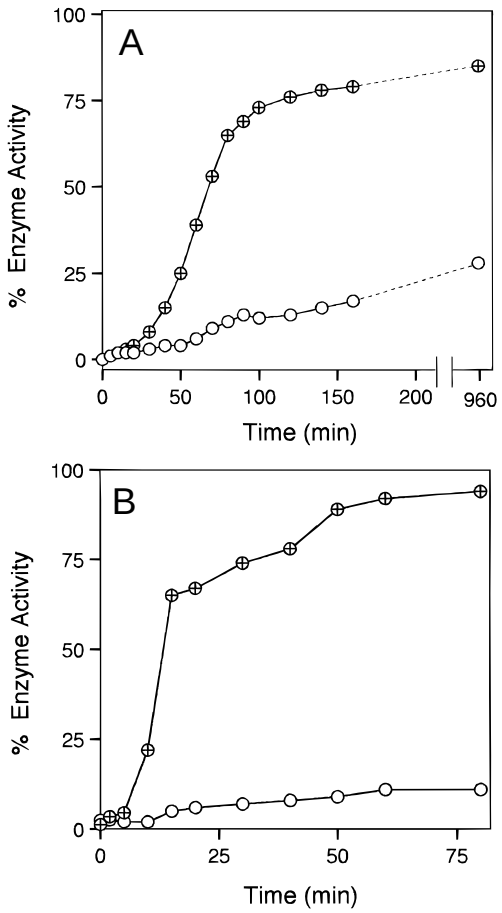

Fig. 4. Protein B23 promotes the renaturation of chemically denatured liver alcohol dehydrogenase and rhodanese. Renaturation was done in the absence (○) or presence (⊕) of equimolar quantities of B23.1 as described in Materials and methods. **A:** LADH. The dashed lines indicate relative activities between the 160 min and 16 h time points. **B:** Rhodanese.

Similar results were obtained when rhodanese was used as a substrate for renaturation (Fig. 4B). In this case only about 10% of the original activity was regained 80 min after dilution of the guanidine HCl in the absence of protein B23. However, the enzyme recovered more than 95% of its activity when incubated with an equimolar quantity of protein B23 for 80 min at room temperature. Thus, protein B23 not only prevents aggregation and protects catalytic activity but it is also able to promote the renaturation of denatured proteins.

Protein B23 preferentially binds denatured substrates

Molecular chaperones generally prefer to bind denatured substrates. To test this possibility, the interaction of protein B23 with native or heat-denatured rhodanese was studied by sedimentation analyses. Samples of protein B23 labeled with ^{125}I were mixed with equimolar quantities of rhodanese and then heated at 65 °C for 60 min. The heated samples and unheated control samples were subjected to analyses by sucrose density gradient centrifugation.

Figure 5A shows that under native conditions there was no interaction between rhodanese and protein B23. However, when the heated mixture was analyzed under the same conditions, the two proteins sedimented together and the enzyme activity of rhodanese was preserved. In control experiments it was found that when rhodanese was heated under the above conditions in the absence of protein B23, no enzyme activity was retained and the protein became insoluble, which precluded its analysis by sedimentation. On the other hand, when protein B23 was heat-treated under the same conditions, its sedimentation profile was nearly identical to that of the untreated sample (not shown). Similar experiments were performed with carbonic anhydrase, with essentially the same results (not shown). Thus, protein B23 appears to preferentially bind denatured proteins.

Hydrophobic regions are exposed in the B23-substrate complex

Compounds based on anilinonaphthalene have increased fluorescence when placed in nonpolar environments, enabling them to be used as probes for hydrophobic regions induced by molecular chaperones (Martin et al., 1991; Mock et al., 1988). To test the possibility that hydrophobic regions are exposed when protein B23 interacts with a denatured substrate, the fluorescence of 1-anilino-8-sulfonate (ANS) was monitored during the interaction of B23 with rhodanese under various conditions. Figure 6 shows that there were only small differences in the fluorescence spectra of ANS in the presence of native or denatured rhodanese. The ANS spectra were also similar when taken in the presence of protein B23 with or without added native rhodanese (Fig. 6, curves 2, 3). However, there was a major increase in fluorescence and a shift toward lower wavelengths when a complex was formed between denatured rhodanese and protein B23 (Fig. 6, curve 1). The ANS fluorescence spectrum in the presence of B23 and denatured rhodanese was stable for several hours (not shown), suggesting that the altered conformation was maintained as long as the complex was not dissociated. Thus, the formation of the complex between denatured rhodanese and protein B23 induces conformational changes resulting in the exposure of hydrophobic regions in one or both of the proteins.

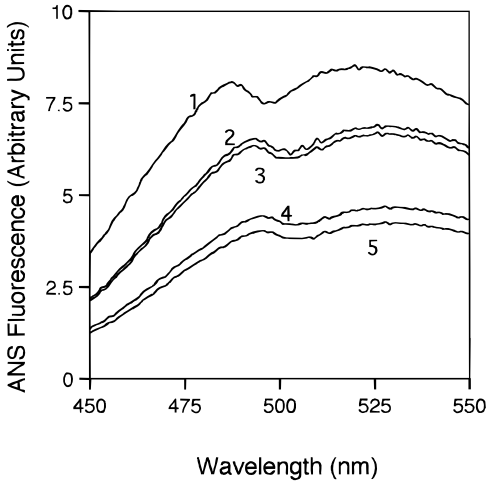

Fig. 6. ANS fluorescence emission spectra of protein B23- substrate complexes. The curve numbers indicate the samples that contained: (1) equimolar concentrations of B23 and denatured rhodanese, (2) equimolar concentrations of B23 and native rhodanese, (3) B23 alone, (4) denatured rhodanese alone, or (5) native rhodanese alone. Curves shown are the average of three emission spectra with excitation at 390 nm.

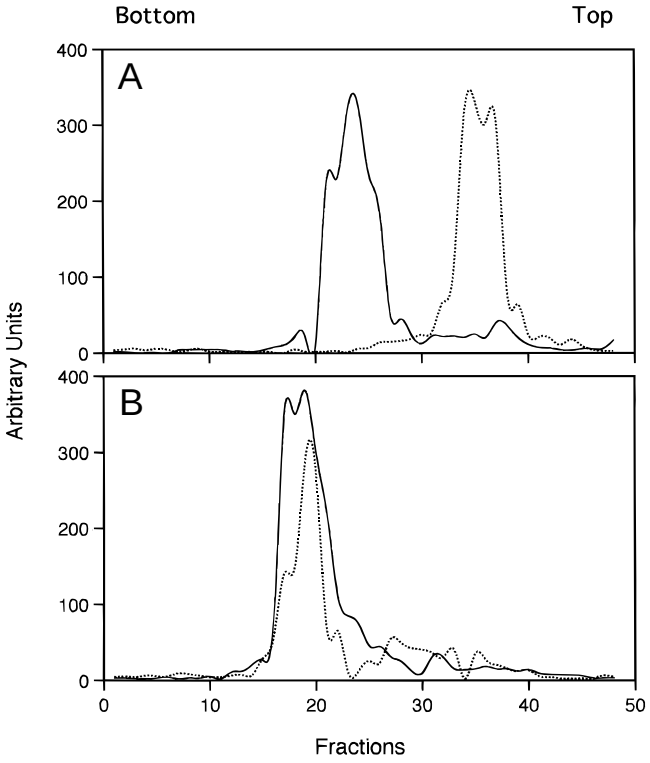

Fig. 5. Sedimentation analysis of ^{125}I labeled protein B23- rhodanese complexes. Equimolar mixtures of protein B23 and rhodanese were subjected to sucrose density gradient sedimentation as described in Materials and methods (**A**) before or (**B**) after 60 min of thermal denaturation at 65 °C. The radioactivity of ^{125}I-labeled protein B23 (solid line) and enzyme activity of rhodanese (dotted line) are indicated.

Discussion

The current studies are the first to show molecular chaperone activities in a nucleolar protein. Protein B23 has several activities that are typically found in chaperones: (1) inhibition of temperature-dependent and -independent aggregation of proteins, (2) suppression of protein aggregation due to thermal denaturation, (3) protection of enzymes against activity loss during thermal denaturation, and (4) promotion of the renaturation of chemically-denatured proteins. Numerous reports in the recent literature have shown that molecular chaperones have one or more of these activities. For example, αB-crystallin is considered to be a molecular chaperone by virtue of its ability to protect LADH against thermal denaturation (Muchowski et al., 1997). The small heat shock proteins, Hsp25 and Hsp27, prevent aggregation under heat shock conditions and promote the refolding of the model substrates citrate synthase and α-glucosidase after chemical denaturation (Jakob et al., 1993). Finally, Hsp90 is able to suppress the aggregation of denatured citrate synthase and enhance its reactivation (Wiech et al., 1992).

Not only does protein B23 have molecular chaperone activities, but the current studies also suggest that the mechanisms of these

activities are similar to those of other chaperones, i.e., preferential binding to denatured substrates and exposure of hydrophobic surfaces. The preference for binding unfolded or non-native substrates is typical of chaperones, e.g., with Hsp90 or the small heat shock proteins (Jakob & Buchner, 1994). The exposure of hydrophobic regions during protein folding is also observed in the chaperonins, including GroEL (Martin et al., 1991) or the eukaryotic equivalent TCP-1 (Norcum, 1996). Thus, it is likely that protein B23 utilizes mechanisms frequently employed by other chaperones.

Most molecular chaperones operate through repeated cycles of binding and release of substrates until the correct conformation is achieved (Buchner, 1996). Typically, the substrate release is coupled with ATP hydrolysis (Hendrick & Hartl, 1995). However, the sedimentation studies presented here indicate that B23-substrate complex is relatively stable, and the substrate is not released under the conditions used. It is interesting that the enzyme activities were also largely preserved in these complexes. This suggests that the proteins bound to protein B23 were sufficiently renatured to maintain nearly complete activity, while still retaining some altered conformation. This is supported by the ANS binding studies showing that the enhanced fluorescence was sustained for several hours in the complexes formed under denaturing conditions. Although recent studies have shown that protein B23 binds ATP (Chang et al., 1998), we have neither observed protein B23-catalyzed hydrolysis of ATP in the presence or absence of substrate, nor seen any effect of ATP on the activities tested. This suggests that in vitro the interaction of B23 with the substrate is governed by simple association-dissociation thermodynamics with no active mechanism for release of substrate as seen with the Hsp70 and chaperonin classes of chaperones (Ruddon & Bedows, 1997). The lack of ATPase activity or energy-dependent release of substrate is typical of Hsp90 and the small heat shock proteins (Jakob & Buchner, 1994), including αB-crystallin (Buchner, 1996). However, it is likely that other unidentified factors or co-chaperones are required for the complete cycle of activity for these chaperones as well as for protein B23.

Although the amino acid sequence of protein B23 bears very little resemblance to other well-characterized chaperones, it does contain segments similar to those found in certain chaperones. For example, Hsp90 contains highly charged regions (Jakob & Buchner, 1994) with some resemblance to similar segments near the center of the protein B23 molecule (Chang & Olson, 1990). In either protein, the function of these highly charged segments is not clear. In protein B23, this portion of the molecule contains two highly acidic regions separated by a positively charged segment. This part of the molecule has been proposed to interact with nuclear localization signals of other proteins (Szebeni et al., 1995, 1997), and it may confer preferential affinity for proteins destined for the nucleus. In addition, the protein B23.1 sequence contains regions of similarity to prolyl cis-trans isomerases, including one found in *Drosophila* (Theopold et al., 1995) and another in yeast nucleoli (Shan et al., 1994). However, in unpublished work, we were unable to show any prolyl cis-trans isomerase activity in recombinant protein B23. Thus, it is difficult to assess the significance of the structural similarities between protein B23 and other chaperones.

What are the physiological roles of the protein B23 chaperone activity? Since protein B23 is predominantly located in the nucleolus, it is likely that its primary functions are performed there. The nucleolus is a very dense organelle (Busch & Smetana, 1970; Hadjiolov, 1984) in which there is a highly crowded environment. A large number of proteins at high concentrations must find their way to specific binding sites without aggregating with themselves or with other proteins. The need for chaperones in the nucleolus is strongly suggested by the earlier observation that the nucleolus is a major location of Hsp70 under conditions of thermal stress, presumably for protection of partially assembled ribosomes and the assembly apparatus (Welch & Feramisco, 1984). Under normal physiological conditions, protein B23 could perform similar functions. It has been shown that protein B23 is associated with preribosomal particles (Olson et al., 1986), and that it interacts with other nucleolar proteins such as nucleolin (Li et al., 1996) and p120 (Valdez et al., 1994). The natural substrates for protein B23 could include these or other nucleolar proteins or ribosomal proteins. Ribosomal proteins tend to be insoluble when they are not contained in ribosomes; i.e., denaturing conditions are required for fractionation or manipulation. Thus, protein B23-ribosomal protein complexes could serve as a transitional phase in the assembly of proteins into ribosomes. Since protein B23 is known to engage in nucleocytoplasmic shuttling (Borer et al., 1989), it is tempting to speculate that the natural substrates combine with protein B23 in the cytoplasm where it could even participate in cotranslational protein folding (Fedorov & Baldwin, 1997). The natural substrates could then be delivered to the nucleolus complexed with protein B23.

A secondary function of protein B23 appears to be related to the nucleolar accumulation of unnatural components such as viral proteins. One of these is the HIV-1 Rev protein, which is essential for regulating the HIV-1 mRNA splicing pattern. The Rev protein is known to localize in the nucleolus and to interact with protein B23 (Fankhauser et al., 1991). The tendency of Rev to aggregate under physiological conditions suggests that it would have difficulty moving about the cell in the absence of interacting molecules. The ability of protein B23 to prevent Rev aggregation may be important in optimizing its mobility and enhancing the efficiency of the virus particle production. Indeed, the importance of chaperone activities in viral function is becoming increasingly clear; e.g., the DnaJ/hsp40 domain of the SV40 large T antigen promotes efficient viral DNA replication (Campbell et al., 1997). It is hoped that future studies will identify the in vivo substrates for protein B23 and elucidate the precise role of its chaperone activities.

Materials and methods

Proteins and peptides

Recombinant proteins B23.1 and B23.2 used in these studies were produced in *Escherichia coli* and purified essentially as previously described (Umekawa et al., 1993) except that the respective cDNAs were inserted into the pET 11c vector (Novagen, Madison, Wisconsin) for expression. The cDNA for the HIV-1 Rev protein was also inserted into pET11c and expressed in *E. coli*. The Rev protein was purified from crude extracts from *E. coli* as described previously (Karn et al., 1995) and refolded as described in Wingfield et al. (1991).

Aggregation assays with the Rev protein

The Rev aggregation assay was based on the observation by Wingfield et al. (1991) that under certain conditions the protein aggregates when the temperature is raised from 0 to 28 °C. The Rev

protein was dissolved in 50 mM phosphate buffer containing 150 mM NaCl, 200 mM KCl, 1 mM DTT, and 1 mM EDTA (pH 7.2), and the protein concentration was adjusted to 0.1 mg/mL. Ice-cold Rev solution was placed in a spectrophotometer cuvette and the temperature was raised from 4 to 28 °C over a 2 h period, during which the absorbance was monitored at 360 nm. For the temperature-independent assay, the Rev protein was dialyzed against 50 mM phosphate buffer containing 1 mM DTT (pH 7.2) at 4 °C in the presence or absence of equimolar quantities of protein B23. The turbidity was monitored at 360 nm over a period of several days.

Thermal denaturation of proteins

Freeze-dried horse liver alcohol dehydrogenase (LADH) was obtained from Fluka (Buchs, Switzerland), and used in an aggregation assay similar to that used with the Rev protein. The aggregation of LADH was monitored by measuring the apparent absorbance at 360 nm in a spectrophotometer at 37 °C. Ice-cold LADH solution (50 μg/mL in 20 mM Tris/HCl buffer, pH 7.4) with or without various concentrations of added protein B23 was placed in the cuvette and the turbidity was recorded automatically for 1 h. Enzyme activity of LADH was assayed in 50 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM NAD and 1 mM ethanol in a final volume of 1 mL and the rate of reduction of NAD^+ was monitored spectrophotometrically at 340 nm according to the method of Guagliardi et al. (1995). Bovine pancreatic carboxypeptidase A (CPA) was obtained from Calbiochem (San Diego, California) as an aqueous crystalline suspension. The crystals were washed according to the instructions provided and then dissolved in 50 mM Tris/HCl (pH 7.5) containing 0.5 M NaCl. The protein concentration was determined with the Bio-Rad protein assay (Bradford, 1976). Thermal aggregation of CPA at a concentration of 4.5 μM was measured as above at 360 nm in a spectrophotometer cuvette held at 48 °C for up to 60 min. Porcine heart citrate synthase (Sigma, St. Louis, Missouri) at 4.5 μM was subjected to thermal denaturation at 43 °C, the course of which was followed by light scattering as described by Buchner et al. (1998).

Denaturation and refolding of LADH and rhodanese

LADH was denatured for 20 min in 6 M guanidine-HCl in 0.1 M phosphate buffer (pH 7.6) containing 0.5 mM DTT and then separated from metal ions on a Sephadex G25 column equilibrated in 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine-HCl and 5 mM EDTA as described by Jaenicke and Rudolph (1990). The denatured protein fraction was used for refolding assays after 100-fold dilution with 0.1 M phosphate buffer (pH 7.6) containing 0.5 mM DTT and 3 μM $ZnCl_2$ at room temperature. Aliquots from the diluted mixture were used to measure the enzyme activity. Rhodanese activity during renaturation subsequent to guanidine HCl denaturation was measured essentially as described by Taguchi and Yoshida (1998). The final concentration of either enzyme was 50 μM during the renaturation experiments.

Sucrose density gradient ultracentrifugation

Protein B23 was labeled with ^{125}I as previously described Szebeni et al. (1997). Rhodanese (250 μg/mL) was incubated with an equimolar concentration of labeled protein B23 at room temperature or at 65 °C for 30 min in 50 mM Tris-HCl buffer (pH 7.8). After incubation the samples were layered onto 5–30% linear sucrose gradients in the same buffer and centrifuged in a Beckman SW41 rotor for 18 h at 38,000 RPM. Fractions (0.5 mL) were collected and the radioactivity of protein B23 and the enzyme activity of rhodanese were measured.

ANS fluorescence measurements

Denaturation of rhodanese was carried out as described by Norcum (1996) with minor modifications. Rhodanese (2 mg/mL) in the presence or absence of protein B23 was denatured in guanidine HCl for 2 h at room temperature prior to taking the fluorescence measurements. The binding of the hydrophobic dye 1,8-ANS was measured using excitation at 390 nm and monitoring the fluorescence emission between 450 and 550 nm.

Acknowledgments

This work was supported in part by NIH grants AI34277 and GM28349. We gratefully acknowledge the helpful discussions with Drs. Miroslav Dundr, Mona Norcum, Laree Hiser, and Rajesh Savkur. We also thank Amy Bauman for preparing the expression constructs for producing B23 and Rev, and Michael Wallace for technical assistance.

References

Borer RA, Lehner CF, Eppenberger HM, Nigg EA. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell 56*:379–390.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem 72*:248–254.

Buchner J. 1996. Supervising the fold: Functional principles of molecular chaperones. *FASEB J 10*:10–19.

Buchner JB, Grallert H, Jacob U. 1998. Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Methods Enzymol 290*:323–338.

Busch H, Smetana K. 1970. *The nucleolus.* New York: Academic Press.

Campbell KS, Mullane KP, Aksoy IA, Stubdal H, Zalvide J, Pipas JM, Silver PA, Roberts TM, Schaffhausen BS, DeCaprio JA. 1997. DnaJ/hsp40 chaperone domain of SV40 large T antigen promotes efficient viral DNA replication. *Genes Develop 11*:1098–1110.

Chan WY, Liu QR, Borjigin J, Busch H, Rennert OM, Tease LA, Chan PK. 1989. Characterization of the cDNA encoding human nucleophosmin and studies of its role in normal and abnormal growth. *Biochemistry 28*:1033–1039.

Chang JH, Lin JY, Wu MH, Yung BYM. 1998. Evidence for the ability of nucleophosmin/B23 to bind ATP. *Biochem J 329*:539–544.

Chang J-H, Olson MOJ. 1990. Structure of the gene for rat nucleolar protein B23. *J Biol Chem 265*:18227–18233.

Eichler DC, Craig N. 1994. Processing of eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol 49*:197–239.

Ellis RJ. 1997. Molecular chaperones: Avoiding the crowd. *Curr Biol 7*:R531–R533.

Fankhauser C, Izaurralde E, Adachi Y, Wingfield P, Laemmli UK. 1991. Specific complex of human immunodeficiency virus type 1 rev and nucleolar protein B23 proteins: Dissociation by the rev response element. *Mol Cell Biol 11*:2567–2575.

Fedorov AN, Baldwin TO. 1997. Cotranslational protein folding. *J Biol Chem 272*:32715–32718.

Feuerstein N, Mond JJ. 1987. Identification of a prominent nuclear protein associated with proliferation of normal and malignant B cells. *J Immunol 139*:1818–1822.

Goldfarb DS. 1988. Karyophilic peptides: Applications to the study of nuclear transport. *Cell Biol Intern Rep 128*:809–832.

Guagliardi A, Cerchia L, Rossi M. 1995. Prevention of in vitro protein thermal aggregation by the *Sulfolobus solfataricus* chaperonin. Evidence for non-equivalent binding surfaces on the chaperonin molecule. *J Biol Chem 270*:28126–28132.

Hadjiolov AA. 1984. *The nucleolus and ribosome biogenesis.* New York: Springer-Verlag.

Hendrick JP, Hartl F-U. 1995. The role of molecular chaperones in protein folding. *FASEB J 9*:1559–1569.

Jaenicke R, Rudolph R. 1990. Folding proteins. In: Creighton TE, ed. *Protein structure a practical approach.* Oxford, UK: IRL Press. pp 191–223.

Jakob U, Buchner J. 1994. Assisting spontaneity—The role of Hsp90 and small Hsps as molecular chaperones. *Trends Biochem Sci 19*:205–211.

Jakob U, Gaestel M, Engel K, Buchner J. 1993. Small heat shock proteins are molecular chaperones. *J Biol Chem 268*:1517–1520.

Karn J, Churcher MJ, Rittner K, Kelley A, Butler PJG, Mann DA, Gait MJ. 1995. RNA binding assays for the regulatory proteins Tat and Rev. In: Karn J, eds. *HIV a practical approach, biochemistry, molecular biology and drug discovery*, Vol. 2. Oxford, UK: IRL Press. pp 147–165.

Li Y-P, Busch RK, Valdez BC, Busch H. 1996. C23 interacts with B23, a putative nucleolar-localization-signal-binding protein. *Eur J Biochem 237*: 153–158.

Martin J, Langer T, Toteva R, Schramel A, Horwich AL, Hartl FU. 1991. Chaperonin-mediated protein folding at the surface of groEL through a molten globule-like intermediate. *Nature 352*:36–42.

Mock DM, Lankford G, Horowitz P. 1988. A study of the interaction of avidin with 2-anilinonaphthalene-6-sulfonic acid as a probe of the biotin binding site. *Biochim Biophys Acta 956*:23–29.

Moss T, Stefanovsky VY. 1995. Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I. *Prog Nucleic Acid Res Mol Biol 50*:25–66.

Muchowski PJ, Bassuk JA, Lubsen NH, Clark JH. 1997. Human B-crystallin. Small heat shock protein and molecular chaperone. *J Biol Chem 272*:2578–2582.

Norcum M. 1996. Novel isolation method and structural stability of a eucaryotic chaperonin: The TCP-1 ring complex from rabbit reticulocytes. *Protein Sci 5*:1366–1375.

Olson MOJ. 1990. The role of proteins in nucleolar structure and function. In: Strauss PR. Wilson SH, eds. *The eukaryotic nucleus: Molecular biochemistry and macromolecular assemblies*, Vol 2. West Caldwell, NJ: Telford Press. pp 541–546.

Olson MOJ, Wallace MO, Herrera AH, Marshall-Carlson L, Hunt RC. 1986. Preribosomal ribonucleoprotein particles are a major component of a nucleolar matrix fraction. *Biochemistry 25*:484–491.

Ruddon RW, Bedows E. 1997. Assisted protein folding. *J Biol Chem 272*:3125–3128.

Schmidt-Zachmann MS, Hugle-Dorr B, Franke W. 1987. A constitutive nucleolar protein identified as a member of the nucleoplasmin family. *EMBO J 6*:1881–1890.

Shan X, Xue Z, Melese T. 1994. Yeast NP146 encodes a novel prolyl cis-trans isomerase that is located in the nucleolus. *J Cell Biol 126*:853–862.

Szebeni A, Herrera JE, Olson MOJ. 1995. Interaction of nucleolar protein B23 with peptides related to nuclear localization signals. *Biochemistry 34*:8037–8042.

Szebeni A, Mehrotra B, Baumann A, Adam SA, Wingfield PT, Olson MO. 1997. Nucleolar protein B23 stimulates the nuclear import of the HIV-1 Rev protein and NLS-conjugated albumin. *Biochemistry 36*:3941–3949.

Taguchi T, Yoshida M. 1998. Chaperonin from thermophile *Thermus thermophilus*. *Methods Enzymol 290*:169–180.

Theopold U, Dal Zotto L, Hultmark D. 1995. FKBP39, a Drosophila member of a family of proteins that bind the immunosuppressive drug FK506. *Gene 156*:247–251.

Umekawa H, Chang JH, Correia J, Wang D, Wingfield PT, Olson MOJ. 1993. Nucleolar protein B23: Bacterial expression, purification, oligomerization and secondary structures of two isoforms. *Cell Mol Biol Res 39*:635–645.

Valdez BC, Perlaky L, Henning D, Saijo Y, Chan PK. 1994. Identification of the nuclear and nucleolar localization signals of the protein p120. *J Biol Chem 269*:23776–23783.

Warner JR. 1990. The nucleolus and ribosome formation. *Curr Opin Cell Biol 2*:521–527.

Welch WJ, Feramisco JR. 1984. Nuclear and nucleolar localization of the 72,000 dalton heat shock protein in heat shocked mammalian cells. *J Biol Chem 259*:4501–4513.

Wiech H, Buchner J, Zimmermann R, Jakob U. 1992. Hsp90 chaperones protein folding in vitro. *Nature 358*:169–170.

Wingfield PT, Stahl SJ, Payton MA, Venkatesan S, Misra M, Steven AC. 1991. HIV-1 Rev expressed in recombinant *E. coli*: Purification, polymerization, and conformational properties. *Biochemistry 30*:7527–7534.

Zimmerman SB, Minton AP. 1993. Macromolecular crowding: Biochemical, biophysical, and physiological consequences. *Ann Rev Biophys Biomol Struct 22*:27–65.