# Sedimentation Analyses of the Salt- and Divalent Metal Ion-Induced Oligomerization of Nucleolar Protein B23[†]

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ABSTRACT: Protein B23 is a major nonribosomal nucleolar protein and putative ribosome assembly factor that has been demonstrated to form oligomers. Sedimentation velocity and equilibrium analyses were used to examine the oligomerization properties of recombinant proteins B23.1 and B23.2. Under low ionic strength conditions protein B23.1 was predominantly a 2.1S monomer with small amounts of a 7.1S oligomer. At NaCl concentrations of 0.1 M and above the protein was almost exclusively the 7.1S oligomer. The oligomer remained the predominant species in NaCl concentrations as high as 1 M, suggesting that oligomers are not stabilized by electrostatic interactions. Low concentrations of divalent metal ions (0.1 − 1 mM $Ca^{2+}$ or $Mg^{2+}$) also promoted oligomerization. Reducing agents had no effect on oligomerization, indicating that disulfide bridges are not important in oligomer formation. Protein B23.2, the carboxyl-terminal truncated isoform, had sedimentation characteristics similar to that of protein B23.1, suggesting that the carboxyl-terminal end of protein B23.1 is not essential for oligomerization. Protein B23.1 was previously shown to bind nucleic acids [Wang, D., Baumann, A., Szebeni, A., & Olson, M. O. J. (1995) *J. Biol. Chem. 269*, 30994−30998]. The effect of protein B23.1 oligomerization on its interaction with a 230 base pair DNA fragment was examined by sedimentation analyses. Under conditions where significant amounts of monomer were present, protein B23.1 was capable of binding DNA, whereas conditions that strongly favored oligomerization caused a nearly complete abolition of DNA binding activity. These studies suggest that protein B23 exists in an equilibrium between monomer and oligomer and that the quaternary structure of the protein may regulate its DNA binding properties.

Protein B23 is an abundant nucleolar protein whose properties suggest that it performs multiple functions in ribosome biogenesis. The ability of protein B23 to shuttle in and out of the nucleolus (Borer et al., 1989) suggests that it participates in the nuclear import process. This is further supported by the fact that protein B23 binds nuclear localization signals (Goldfarb, 1988; Szebeni et al., 1995) and proteins containing them, e.g., the HIV-1 Rev protein (Fankhauser et al., 1991). The predominant location of protein B23 in the nucleolus (Spector et al., 1984) implies that the proposed transport function may be directed toward ribosomal proteins which are being incorporated into ribosomes in the process of assembly. Protein B23 has also been shown to bind RNA and single- and double-stranded DNA (Dumbar et al., 1989; Feuerstein et al., 1990; Wang et al., 1994). This nucleic acid binding property has been suggested to be the basis for nucleolar localization (Wang et al., 1994), and it may be involved in regulation of nucleolar DNA synthesis (Feuerstein et al., 1990) and ribosome assembly (Dumbar et al., 1989). Finally, the protein has recently been demonstrated to have intrinsic ribonuclease activity (Herrera et al., 1995). This ribonuclease activity is modulated by ionic strength as well as by divalent metal ions.

By cDNA analyses protein B23 has been shown to exist as two different isoforms designated as B23.1 and B23.2 with molecular masses of 32.6 and 28.4 kDa, respectively (Chang & Olson, 1989). However, under native conditions several laboratories have observed that protein B23 has an apparent molecular mass of 230−255 kDa, suggesting that the protein exists as a hexamer (Schmidt-Zachmann et al., 1987; Yung & Chan, 1987; Umekawa et al., 1993). Recent studies by Chan and Chan (1995) using native gel electrophoresis suggested that the protein exists largely as a hexamer in cells. However, studies by Umekawa et al. (1993) and preliminary work in this laboratory have indicated that a significant portion of protein B23 is found as a lower molecular weight species. Therefore, we searched for conditions that would promote either the monomeric or the oligomeric form of the protein. In the current study we used analytical ultracentrifugation to examine the oligomerization properties of the protein under varied conditions of ionic strength and divalent metal ions. We observed that low ionic strength and the absence of divalent metal ions promote monomer formation whereas increased ionic strength or the presence of divalent metal ions promotes oligomerization. Furthermore, using the same methods, it was shown that the DNA binding activity of the protein is optimal under conditions that promote monomer formation. These results suggest that some functional properties of the protein are regulated by the monomer−oligomer equilibrium.

## EXPERIMENTAL PROCEDURES

*Proteins, Nucleic Acids, and Chemicals*. The recombinant forms of rat proteins B23.1 and B23.2 were purified as

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction.

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previously described (Umekawa et al., 1993) except that the cDNAs for each were inserted into the pET11c protein expression vector (Novagen). All reagents were purchased as ACS reagent grade. DNA used in this study was a 230 bp fragment containing nucleotides −98 to +132 of a gene for rat preribosomal RNA generated by PCR amplification of a segment of plasmid p8.5 (Cassidy et al., 1986). The PCR products were purified using a Wizard PCR purification kit (Promega), and the purity was verified on agarose gels.

*Analytical Ultracentrifugation*. All experiments were performed on a Beckman Optima XLA analytical ultracentrifuge equipped with absorbance optics and an An60Ti rotor. Temperature was calibrated as previously described by Lui and Stafford (1995). All experiments were performed at 25 °C which was found to correspond to 24.6 °C (unless otherwise indicated). Sedimentation velocity studies were done at 42 000 rpm in charcoal-filled epon double-sector centerpieces. Velocity data were collected at 280 nm and at a spacing of 0.01 cm with four averages in a continuous scan mode. All studies were performed in 10 mM Tris-HCl (pH 7.0) with or without added effectors and with protein concentrations between 6 and 60 $\mu$M, ranging in $OD_{280}$ from 0.1 to 1.0. The protein samples were dialyzed into 10 mM Tris-HCl (pH 7.0) with or without the concentrations of NaCl, $MgCl_2$, or $CaCl_2$ as indicated in the Results section. The proteins were subjected to ultracentrifugal analyses with the dialysate in the reference cell. The velocity data were analyzed using SVEDBERG (John Philo, Amgen, Inc.) and DCDT (Walter Stafford, Boston Biomedical Research Institute). SVEDBERG uses Faxen's approximation of the Lamm equation to fit the absorbance profiles from the velocity runs to sedimentation and diffusion coefficients for up to three non-interacting species (Philo, 1994). DCDT generates a distribution of sedimentation coefficients, $g(s)$, by taking the differences in absorbance profiles of successive timed scans, averaged over all differences (Stafford, 1992a,b). Sedimentation coefficients were corrected to standard conditions, $s_{20,w}$, where indicated (Laue et al., 1992). Equilibrium experiments were performed at 8 000 and 13 000 rpm in charcoal-filled epon six-channel centerpieces. Equilibrium data were collected at 280 nm and at a spacing of 0.001 cm with 16-scan averaging in a step mode. Equilibrium was verified by examining scans at time intervals up to 20 h. Data sets were edited with reedit (Jeff Lary, National Analytical Ultracentrifuge Center, Storrs, CT) to extract and fit individually with NONLIN (Johnson et al., 1981). Fits to single species yield a *Z*-average $\sigma$ value and thus a *Z*-average molecular weight (Lansing & Kramer, 1935). Solvent densities and viscosities were calculated according to Laue et al. (1992). The partial specific volumes for the two isoforms B23.1 and B23.2 were calculated to be 0.7246 and 0.7221, respectively, using the method of Laue et al. (1992).

*Filter Binding Assay*. Binding of the 230 bp DNA fragment to recombinant protein B23.1 under various conditions of ionic strength or divalent metal ion content was quantified by the nitrocellulose filter binding assay essentially as described previously (Wang et al., 1994). In the current work the binding buffer was 10 mM Tris (pH 7.0) containing one of the following: 0.1 M NaCl, 0.1 mM $CaCl_2$, or 1.0 mM $MgCl_2$.

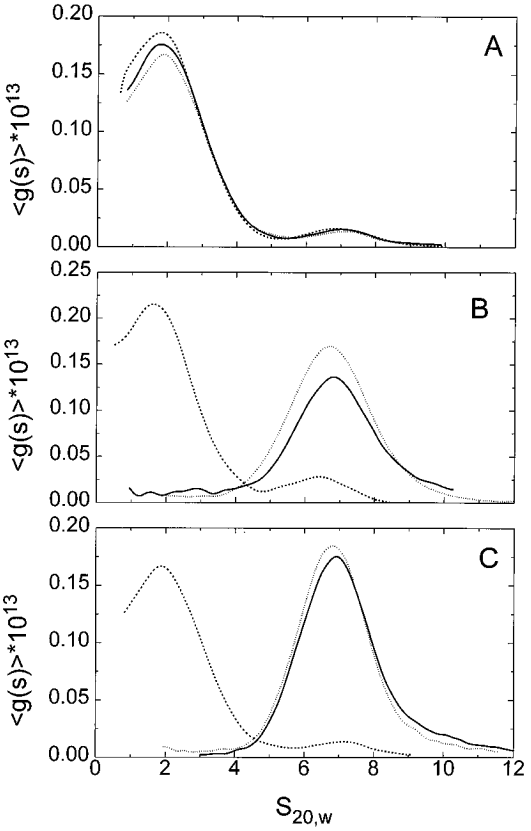


FIGURE 1: Sedimentation coefficient distribution analyses of protein B23.1 under various conditions. Prior to ultracentifuge analyses all samples were dialyzed against 10 mM Tris (pH 7.0) containing different salts, divalent metal ions or reducing agents. The concentration of B23.1 was 25−29 $\mu$M. The data were collected as described in the Experimental Procedures section. The sedimentation distribution, $g(s)$, plots were generated as described in Materials and Methods using DCDT with the S values corrected to $s_{20,w}$. (A) $g(s)$ plots for samples centrifuged in the presence of no DTT (dashed line), 0.1 mM DTT (solid line) and 1.0 mM DTT (dotted line). (B) $g(s)$ plot for samples centrifuged in the presence of no added effectors (dashed line), 0.1 mM $CaCl_2$ (dotted line), or 0.1 mM $MgCl_2$ (solid line). (C) Effect of NaCl on the sedimentation coefficient distribution. Protein B23.1 was centrifuged in the presence (solid line) or absence (dashed line) of 0.1 M NaCl or in the presence of 0.1 M NaCl plus 0.1 mM DTT (dotted line).

## RESULTS

*Effects of Mono- and Divalent Metal Ions on B23.1 Oligomerization.* To determine conditions that promote monomer or oligomer formation, protein B23.1 was subjected to sedimentation velocity analyses in the presence or absence of various concentrations of NaCl or divalent metal ions. The $g(s)$ analysis (Figure 1A) showed that at low ionic strength (10 mM Tris) and in the absence of added divalent metal ions, protein B23.1 existed in two forms, a major slowly sedimenting form and a minor faster sedimenting form. The major and minor forms had sedimentation coefficients of 2.1 and 7.1 S, respectively. Analysis of the data using SVEDBERG indicated that more than two species may be present, suggesting an equilibrium between the two prominent species with intermediates as well as larger species that are not readily detectable (data not shown). The component sedimenting at 2.1 S was assumed to be a monomer since this was the smallest species detected and analyses of sedimentation equilibrium measurements indicated an equilibrium between a 32 kDa species, correspond-

ing to a single polypeptide chain, and a hexamer (data not shown).

Several conditions were examined to determine conditions that promoted formation of the oligomers. Initially, the effects of dithiothreitol were investigated to determine the possible involvement of disulfide bond formation during oligomerization. Figure 1A shows the effects of dialysis of protein B23.1 against 0.1 and 1.0 mM DTT. Inclusion of DTT had no effect on the ratio of monomer to oligomer, suggesting that disulfide bonds are not involved either in formation of the oligomer or in maintaining an equilibrium between the two forms of the protein.

Because divalent metal ions often promote the oligomerization of proteins the effects of $Ca^{2+}$ and $Mg^{2+}$ were examined. Figure 1B shows that both $CaCl_2$ and $MgCl_2$ induced oligomerization of protein B23.1 to the 7.1S oligomer at a concentration of 0.1 mM. Under either condition little or none of the 2.1S species was detectable. Further addition of $MgCl_2$ up to 1 mM had no effect on the sedimentation properties (data not shown). However, addition of $CaCl_2$ up to 1 mM resulted in precipitation of the protein.

The ionic strength dependence of the oligomerization was examined by the addition of NaCl. Figure 1C shows that the addition of 0.1 M NaCl resulted in nearly complete conversion to the 7.1S oligomer. Under these conditions no detectable 2.1S species was observed. Addition of NaCl up to 1M had no effect on the observed distribution (data not shown). In order to assure that the oligomer was not stabilized by formation of disulfides we tested whether the NaCl induced oligomer could be reversed by the addition of 0.1 mM DTT. Figure 1C shows that addition of DTT after oligomerization by NaCl did not alter the observed distribution of sedimentation species. Removal of salt by dialysis resulted in essentially complete conversion to the 2.1S species (data not shown), suggesting that the oligomerization process is a reversible equilibrium.

*Oligomerization of Protein B23.2.* Protein B23 exists as two isoforms, B23.1 and B23.2 (Chang & Olson, 1989). The two proteins differ only in their C-terminal sequences with the N-terminal 255 residues being identical in both forms. Beyond the point of identity there are 37- and 2-residue extensions in B23.1 and B23.2, respectively. Experiments were performed to determine whether the B23.2 isoform exhibited oligomerization properties similar to the longer isoform. The distribution plots in Figure 2 show that B23.2 also exists as two forms in low ionic strength buffer. In this case the sedimentation coefficients were slightly smaller than those for B23.1: 1.8 and 6.8 S for the monomer and oligomer, respectively, reflecting the lower molecular weight of the B23.2 isoform. Under low ionic strength conditions the proportion of oligomer was also slightly higher than that seen with B23.1 (compare with the distribution in Figure 1A). Dialysis against 0.1 M NaCl resulted in nearly complete conversion of the protein to the oligomeric species of 6.8 S (Figure 2). Addition of 0.1 mM $CaCl_2$ also shifted the equilibrium predominantly to the 6.8S oligomer; however, a considerable amount of 1.8S monomer remained under these conditions. Thus, the oligomerization behavior of protein B23.2 was generally similar to that of B23.1 although shifts to either monomer or oligomer were not as complete under the same conditions.

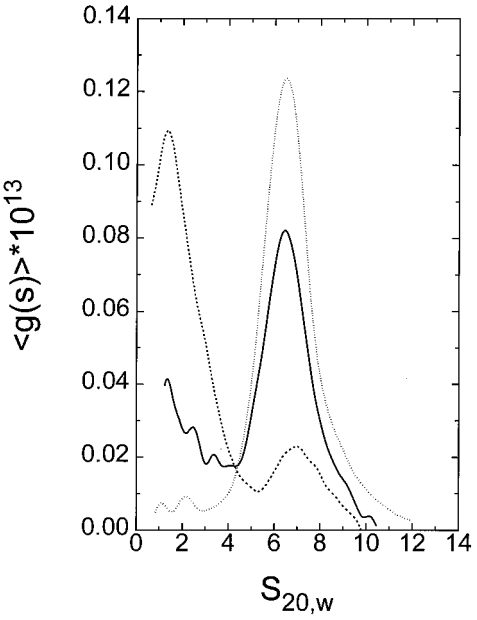


FIGURE 2: Sedimentation coefficient distribution analyses of protein B23.2 under various conditions. Samples were dialyzed against 10 mM Tris (pH 7.0) in the absence (dashed line) or presence of 0.1 M NaCl (dotted line) or 0.1 mM $CaCl_2$ (solid line). The studies were performed at $OD_{280}$ of 0.5 (0.5 mg/mL). Protein B23.2 does not contain tryptophan and thus has a lower extinction coefficient than the B23.1. Therefore, the molar concentration of B23.2 in these studies was greater than that used with the B23.1 isoform to allow for the same absorbance value at 280 nm. The *g*(*s*) plots were generated using DCDT as described in Figure 1.

*Sedimentation Equilibrium.* Equilibrium studies were conducted in the presence of low salt, high, salt and divalent cations. The data were consistent with an equilibrium between monomer and hexamer that shifts to predominantly hexamer at higher ionic strength or in the presence of $Ca^{2+}$ or $Mg^{2+}$. However, quantitative fitting of the data was complicated by the presence of higher molecular weight species of 10 S or greater (see Figures 1−4). These aggregates do not negate the conclusions of this study, but they do preclude extraction of equilibrium constants for the monomer−hexamer association.

*DNA Binding Properties of the B23.1 Oligomer and Monomer.* Since the equilibrium between oligomer and monomer could be shifted by the addition of either mono- or divalent ions, we examined the possibility that a functional property of the protein was affected by the oligomerization. Protein B23.1 has been demonstrated to bind DNA (Wang et al., 1995); therefore we investigated the effect of oligomerization on its DNA binding activity. Because of limitations of the optical detection system of the ultracentrifuge, lower concentrations of protein than those employed for protein alone were used to accommodate the optical contribution of the added DNA. Because of these optical limitations, it was also necessary to use concentrations of protein B23.1 that were above saturating the DNA. Figure 3 shows the sedimentation behavior of the protein or DNA alone and the mixtures of the two in the presence or absence of NaCl. As also indicated above, low ionic strength conditions resulted in an equilibrium between monomer and oligomer in which the monomer was strongly favored. Figure 3A shows that at low ionic strength the DNA (5.8 S) is essentially completely shifted to a 7.2S species when an excess of protein B23.1 is present. This indicates that under

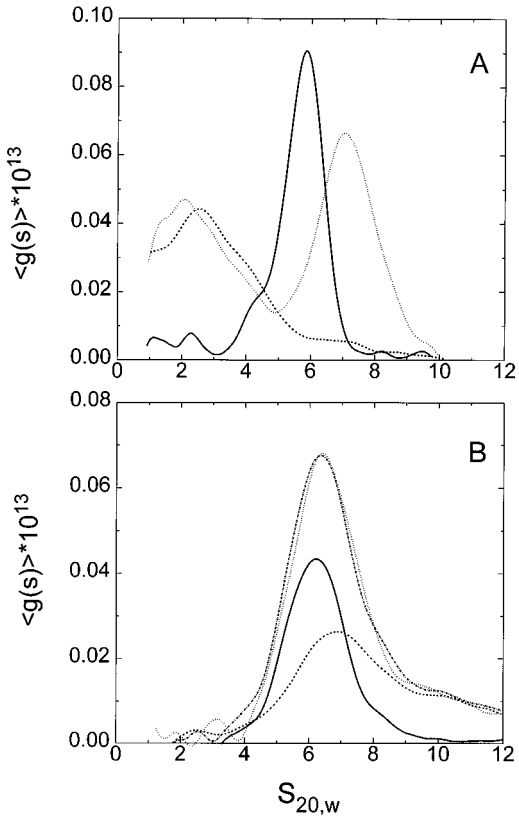


FIGURE 3: Effect of NaCl on the DNA binding activity of protein B23.1. Protein B23.1 (dashed line), a 230 bp fragment of DNA (15 $\mu$M in bp, solid line) or the mixture of protein B23.1 (18 $\mu$M) and the DNA (15 $\mu$M bp, dotted line) were subjected to sedimentation velocity analyses as described in the Experimental Procedures. (A) $g(s)$ plots of B23.1, DNA, and the mixture of the two in the absence of added NaCl. The conditions in panel B were identical to those in panel A except that the buffer contained 0.1 M NaCl. The dot−dash line in B is the numerical sum of the $g(s)$ plots for protein alone and DNA alone.

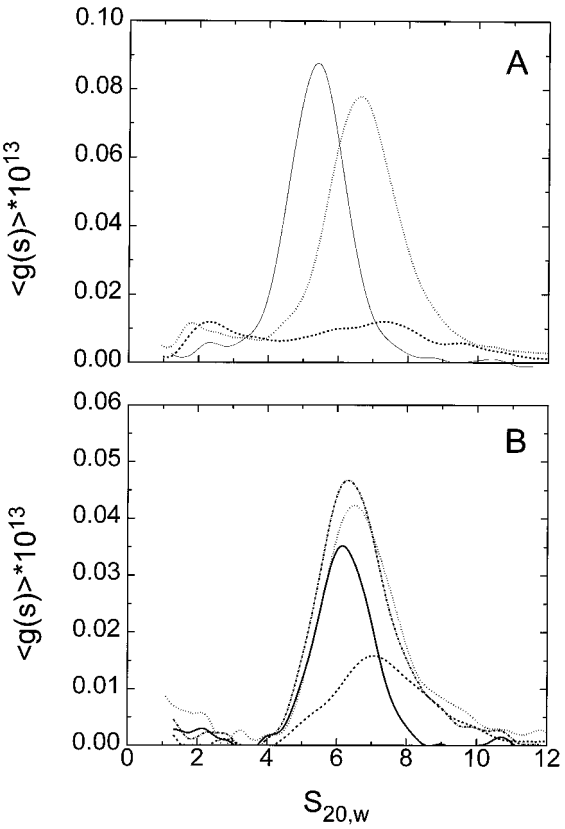


FIGURE 4: Effects of divalent metal ions on the DNA binding activity of protein B23.1. Protein B23 (7.0 $\mu$M, dashed line) or a 230 bp fragment of DNA (10 $\mu$M in bp, solid line) or a mixture of protein B23.1 and the DNA at the same concentrations (dotted line) were subjected to sedimentation velocity analyses as described in the Experimental Procedures. (A) $g(s)$ plots for sedimentation analyzed performed in 10 mM Tris (pH 7.0) containing 0.1 mM $CaCl_2$. The conditions in panel B are the same as in panel A except that the buffer contained 1.0 mM $MgCl_2$ instead of 0.1 mM $CaCl_2$. Dash−dot line, sum of the DNA-alone and protein-alone plots.

these conditions the protein saturates the DNA so that there is a quantitative shift to the faster sedimenting species.

The above experiments suggested that the monomer form of B23.1 bound DNA since the monomer was the predominant species at low ionic strength. However, these experiments were not capable of determining whether the oligomer also bound DNA since the binding could shift the equilibrium to the active species. To assess whether the oligomer bound DNA, we examined the sedimentation behavior of the mixture of DNA and protein under conditions that strongly favored oligomer formation. Figure 3B shows that the addition of 0.1 M NaCl, which causes a nearly complete shift to the B23.1 oligomer, resulted in no shift of the DNA to the 7.2S species. Further, the sum of the distribution plots for DNA and protein alone superimposes with the $g(s)$ plot obtained from the mixture of the two. These data indicate that when protein B23.1 and DNA were mixed in 0.1 M NaCl each component sedimented independently. Thus, the oligomer apparently does not bind DNA, implying that only the monomeric form of the protein is the active species in DNA binding. These data also suggest that the free energy of B23.1 oligomer formation is greater than the free energy of B23.1 binding to DNA since the presence of DNA does not shift the equilibrium.

Additional experiments were done to rule out the possibility that the increase in ionic strength rather than oligomer formation reduced the DNA binding activity. As shown above, the presence of either $Mg^{2+}$ or $Ca^{2+}$ shifts the equilibrium toward the oligomer without significantly increasing the ionic strength. Figure 4 shows the effects of these divalent metal ions on DNA binding. Addition of $CaCl_2$ resulted in a shift in the equilibrium toward the oligomer (Figure 4A). However, using the combination of a relatively low concentration of protein (7 $\mu$M) and 0.1 mM $CaCl_2$ a sizable amount of the protein was detected as a monomer. Under these conditions, protein B23.1 was able to bind and shift the DNA to the 7.2S species (Figure 4A). Thus, DNA binding occurred when monomer was present.

Since the above conditions caused only a partial shift to the oligomer we examined conditions that would essentially quantitatively produce oligomers at low ionic strength. Figure 4B shows that in the presence of 1 mM $MgCl_2$ no detectable amounts of monomer existed. Under these conditions there was no shift of the DNA peak, indicating that protein B23.1 did not bind DNA. Furthermore, the sum of the distribution plots for protein alone and DNA alone is virtually superimposable with the distribution profile observed for the mixture of the two, suggesting that DNA and protein are sedimenting as independent species and, therefore, no protein−DNA complex is forming.

To verify that conditions favoring monomer formation also promote DNA binding activity, traditional nitrocellulose filter binding assays were performed under the same conditions

employed in the ultracentrifuge experiments. In these experiments, maximum DNA binding was seen in the presence of 0.01 M Tris buffer with no added salt (data not shown), a condition which promotes monomer formation. Significant DNA binding activity was also observed in the presence of 0.1 mM $CaCl_2$, which produced a mixture of monomer and oligomer (see above). However, when either 1 mM $MgCl_2$ or 0.1 M NaCl was added to the binding buffer, the DNA binding activity was only slightly above background. As shown in the sedimentation experiments the latter conditions promote nearly a complete shift to the oligomer form of the protein. Thus, the filter binding results parallel the behavior observed in the ultracentrifuge studies.

## DISCUSSION

The studies reported here demonstrate that protein B23 exists in an equilibrium between two predominant forms: a 2.1S monomer and a 7.1S oligomer. Conditions were found which could shift this equilibrium to favor either of the two forms. On the one hand, low ionic strength and absence of divalent metal ions favor monomer formation. On the other hand, the presence of either monovalent ions above 0.1 M or divalent metal ions as low as 0.1 mM promotes oligomerization. Protein B23 is highly negatively charged because of its high content of acidic amino acids. Under low ionic strength conditions the repulsive forces between the negatively charged molecules could promote monomer formation. However, electrostatic interactions do not appear to be the dominant forces in stabilizing the oligomers since high concentrations of NaCl did not cause dissociation. Thus, it seems likely that conformational changes induced by salt or divalent ions enable the protein−protein interactions required for oligomer formation.

Sedimentation studies with protein B23.2 indicate that this protein has oligomerization properties virtually identical to those of B23.1. Since proteins B23.1 and B23.2 differ only in their carboxyl-terminal ends, this segment must not be essential for oligomerization.

Several laboratories have previously observed that protein B23 is capable of oligomerization and probably exists as an oligomer in cells. High molecular weight forms of the protein which persisted under denaturing conditions were originally seen by Fields et al. (1986) and by Yung and Chan (1987). Under native conditions the molecular mass of the protein has been estimated to be 230−255 kDa (Schmidt-Zachmann et al., 1987; Yung & Chan, 1987; Umekawa et al., 1993) which approximately corresponds to a hexamer of B23. Finally, Chan and Chan (1995) concluded that the B23 oligomer is the predominant stable entity in cells. The aforementioned *in vitro* studies were conducted in the presence of monovalent ions at approximately 100 mM or in the presence of divalent metal ions in the range of 1−2 mM. Furthermore, cells contain monovalent cations in the 140−160 mM range and $Mg^{2+}$ at about 0.5 mM (Alberts et al., 1994). The current work indicates that all of the these conditions strongly promote oligomer formation. Knowing this, it is not surprising that earlier reports concluded that the oligomer is the major form of the protein. Thus, in the nucleolus, protein B23 would be expected to be largely in the oligomer form.

A surprising finding of this study was that the DNA binding activity of protein B23.1 appears to be modulated by its quaternary structure. Under conditions where monomer was favored (e.g., low ionic strength) the protein bound DNA. However, in the presence of 0.1 M NaCl or 1 mM $Mg^{2+}$, which strongly induced oligomerization, the protein did not bind DNA. These results were independently confirmed using DNA filter binding assays. Although the DNA binding activity strongly correlates with the presence of monomer, the reduced DNA binding activity seen in 0.1 M NaCl or 1 mM $Mg^{2+}$ could possibly be due to ion shielding. Even though 1 mM $MgCl_2$ does not markedly elevate the ionic strength, $Mg^{2+}$ ion is more effective at shielding DNA−protein interactions than $Na^+$ ion (Record *et al*., 1977). Therefore, it cannot completely be ruled out that the reduced DNA binding activity is due to ion shielding rather than oligomerization, although this seems unlikely.

Protein B23 binds both RNA and DNA (Dumbar et al., 1989; Feuerstein et al., 1990; Wang et al., 1994), is found in preribosomal RNP particles (Schmidt-Zachmann et al., 1987), and is associated with the nuclear matrix (Feuerstein & Mond, 1987). However, the precise cellular or nucleolar target nucleic acid sequences have not been identified. It could be argued that conditions in cells are such that only oligomer is present, and hence, there is no binding of nucleic acids by protein B23. However, in the current study protein was always in a large excess over DNA. This situation is unlikely in the cell, where DNA or RNA are in excess over protein B23. The cellular excess of nucleic acid relative to protein B23 could shift the equilibrium toward formation of the monomer and thus promote nucleic acid binding. Furthermore, the current work indicated that small amounts of monomer were present even under conditions that favored oligomer formation (not shown). In addition, Chan and Chan (1995) showed that small but significant amounts of monomer are present in cells. Therefore, it is likely that some protein B23 binds nucleic acids in cells. The oligomerization process may be a way of modulating the nucleic acid binding activity of the protein. In addition, the monomer−oligomer equilibrium could be responsible for the cooperativity observed in B23 binding to oligoribonucleotides (Dumbar et al., 1989).

In recent studies, Herrera et al. (1995) showed that the ribonuclease activity of protein B23 is optimal at a salt concentration of about 0.1 M and is stimulated by mM concentrations of $Ca^{2+}$ and $Mg^{2+}$. Curiously, these are conditions that promote oligomerization and diminish the DNA binding activity of protein B23. Thus, the ribonuclease and nucleic acid binding activities appear to have opposite responses to shifts in the equilibrium between monomer and oligomer. It is tempting to speculate that the ribonuclease activity can be modulated by the sequestering of the protein onto DNA, which in turn is regulated by the monomer−oligomer equilibrium. Further studies will be required to determine the interplay between the ribonuclease and nucleic acid binding activities and how these are affected by the monomer to oligomer ratios *in vivo*.

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