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Stoichiometries of Protein−Protein/DNA Binding and Conformational Changes for the Transition-State Regulator AbrB Measured by Pseudo Cell-Size Exclusion Chromatography−Mass Spectrometry†

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ABSTRACT: We have developed on-line pseudo cell-size exclusion chromatography−mass spectrometry (PsC-SEC−MS) for the rapid, real time analyses of noncovalently bound protein complexes. The methodology can be used to determine constituent components of such complexes, as well as exact stoichiometries. Furthermore, it enables the efficient determination of gross conformational changes upon complexation. The power of the new approach is demonstrated in the analysis of the global transition-state regulator AbrB and its complex with a target DNA sequence from the promoter *sinIR*. Using PsC-SEC−MS, we confirm that AbrB is assembled as a homotetramer and not as a homohexamer as previously suggested. Additionally, we show that AbrB binds to the *sinIR* DNA target element as a homotetramer, affording a 4:1 protein:DNA stoichiometry. Finally, we demonstrate that when the complex binds to *sinIR*, the hydrodynamic volume (size) of the complex is notably reduced compared to that of the apoprotein, indicating a protein conformational change.

Global transition-state regulators are emerging as a critical and discrete class of proteins. To this point, ~20 transition-state regulators have been definitely or potentially identified in a myriad of organisms, including *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus anthracis*, *Clostridium acetobutylicum*, *Mycobacterium tuberculosis*, and *Salmonella typhimurium*. They are known to exert wide-ranging regulatory control during postexponential growth and play an essential role in the adaptive and survival capacity of the organism (*1*−*3*). To date, a great deal of effort has focused on the genetic characterization of transition-state regulators, while little is known about their structure and function at the molecular level. The *B. subtilis* transition-state regulator AbrB is known to control more than 60 different genes nominally expressed or repressed in suboptimal environments characterized by limited nutrients. It is a multimeric protein with each monomer consisting of 94 amino acids (~10.5 kDa). Previous studies have shown that the monomer consists of a C-terminal domain (residues 54−94) that is involved

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in AbrB multimeric assembly (*4*). The N-terminal domain (residues 1−53) is involved in both DNA binding and multimerization (*4*, *5*). DNase I footprinting has revealed that there is no obvious DNA consensus sequence of AbrB−promoter elements that recognize AbrB (*6*, *7*). However, other studies have shown that AbrB binding is specific and generally occurs in regions up- or downstream of the target gene +1 initiation site (*8*). It has been suggested that DNA targeting by AbrB may depend on recognition of a general DNA tertiary structure (*5*, *8*). Previously, AbrB has been inferred to exist functionally as a homohexamer (*8*, *9*). In both cases, indirect measurements were made utilizing either nondenaturing gels or gel filtration chromatography with UV detection. It has also been reported that a number of sequence specific double-stranded oligonucleotide sequences bound AbrB (*10*). All these latter measurements were taken using a gel retardation assay.

The functional role of AbrB within *B. subtilis* utilizes important noncovalent interactions involving both protein−protein and protein−DNA complexes. Such complexes can be somewhat transient, and the interactions between components are often characterized by low affinity (micromolar to nanomolar) binding constants. Hence, any technique that can rapidly delineate any aspects of structure and stoichiometry and identify constituent components of such biological complexes would be invaluable. Numerous methods have already been described and include NMR, various spectroscopic approaches such as fluorescence, circular dichroism, surface plasmon resonance, nondenaturing electrophoretic approaches, and analytical ultracentrifugation (*11*−*13*). All these methods have different measured end points, and all possess well-known inherent advantages and disadvantages. An ideal technique would be one that allows the examination of noncovalent biological interactions without perturbing the complex and is also nondestructive (or at least sparing of material used), specific, sensitive, and reproducible and provides rapid, real-time analysis. To this end, we have developed a new approach that addresses such issues. In this work, we describe the use of on-line pseudo cell-size exclusion chromatography−microelectrospray ionization mass spectrometry (PsC-SEC−μESI-MS)[1] to carry out functional analyses on noncovalent complexes of the transition-state regulator protein AbrB. Electrospray ionization mass spectrometry (ESI-MS) is finding increasing use in the analysis of noncovalent complexes (*14*−*16*).

The PsC-SEC−μESI-MS platform technology was developed to be a general procedure for efficiently separating and introducing complex biological mixtures directly into the mass spectrometer without any additional sample handling. The latter process always results in significant sample losses (*17*). Furthermore, it was important to ensure that any chromatography step that was used actually performed two important, but distinct, functions. First, it should allow the removal of high levels of nonvolatile salts from the biological mixture immediately prior to analysis by mass spectrometry. Second, the separation of constituents should not dissociate any noncovalent complexes already present in the biological mixture.

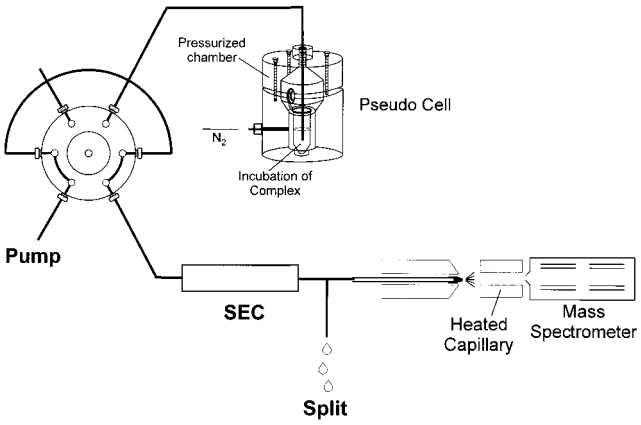

FIGURE 1: Schematic of PsC-SEC−MS. The system consists of a pseudo cell made out of polyacrylamide with multiple inlet ports (not shown for clarity). A pneumatic outlet allows a sample to be collected in a microliter capillary. Samples are separated on a 0.8 mm × 150 mm Superdex 200 size exclusion capillary column. The sample is split with ~300−500 nL/min of eluent flowing into the low-flow microelectrospray source.

To achieve this capability, we devised the PsC-SEC−MS arrangement shown schematically in Figure 1. It consists of a "pseudo cell" (PsC), which is used as an incubation and sample delivery system. The PsC is a polyacrylamide chamber that contains a microcentrifuge tube. Multiport entry (not shown for clarity) is available for adding potential substrates and ligands such as peptides, metal ions, other proteins, nucleic acids, or biopolymers. Biological samples are placed in the microcentrifuge tube and incubated for a defined period of time. A sample aliquot is then pneumatically loaded into a microliter sample loop configured on a multiport valve. The SEC capillary column separates analytes on the basis of their hydrodynamic molecular volume or size (*18*). Since the mechanism of separation involves analyte−gel pore interactions, noncovalent complexes are not dissociated in the separation process. Furthermore, SEC is a very efficient method for removing nonvolatile salts and buffers prior to mass spectrometric analysis. The coupling of PsC-SEC to microelectrospray ionization mass spectrometry (μESI-MS) allows direct, specific, and sensitive analysis of any complexes present in a biological mixture, which contains high salt concentrations which are often necessary for complex formation and stability. This approach affords minimal sample use and maximum specificity and sensitivity and allows ready determination in changes in the size of complexes relative to individual components.

MATERIALS AND METHODS

Expression and Purification of AbrB. The AbrB vector construct was provided by M. Strauch (OCBS Department, Dental School, University of Maryland, Baltimore, MD). DNA was isolated using a Wizzard prep kit from Promega. The plasmids were transformed into competent *Escherichia coli* JM109 cells purchased from Strategene. One liter of LB broth containing 50 μg/mL carbenicillin was inoculated and grown at 37 °C to an optical density (OD) A_{600} of ~0.700. IPTG (isopropyl β-D-thiogalactopyranoside) was

[1] Abbreviations: PsC-SEC−MS, pseudo cell-size exclusion chromatography−mass spectrometry; PsC-SEC−μESI-MS, pseudo cell-size exclusion chromatography−microelectrospray ionization mass spectrometry; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; SEC, size exclusion chromatography; hEFT, recombinant human protein; BSA, bovine serum albumin; PsC, pseudo cell; μESI-MS, microelectrospray ionization mass spectrometry; PMSF, phenylmethanesulfonyl fluoride; βME, β-mercaptoethanol.

added to a final concentration of 1 mM and the incubation continued for ~2−3 h. The cells were pelleted by centrifugation and resuspended with 10 mM Tris-HCl (pH 8.3 at 4 °C or pH 7.9 at room temperature), 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM KCl, 10 mM β-mercaptoethanol (βME), and 0.25 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). All subsequent steps were performed at 4 °C. The cells were sonicated for five cycles of 45 s bursts and 2 min rests. The resulting suspension was again centrifuged at 17 000 rpm for 25 min. The supernatant was removed and saved as the crude extract. The crude extract was dialyzed into 10 mM Tris-HCl (pH 8.3 at 4 °C or pH 7.9 at room temperature) and 10 mM KCl. Any residual DNA or RNA was removed by incubating with S7 nuclease for 15−30 min at room temperature. The reaction was quenched by dialyzing into EDTA. Solid ammonium sulfate was added slowly to the supernatant to a final concentration of 55%. This was allowed to sit for 30 min and was then recentrifuged at 17 000 rpm. The pellet was checked for protein and subsequently discarded. The supernatant was dialyzed into 10 mM Tris-HCl (pH 8.3 at 4 °C or pH 7.9 at room temperature), 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM KCl, and 10 mM βME. The dialyzed supernatant containing AbrB was purified via column chromatography using DEAE-Trisacryl. AbrB does not bind and elutes off during the wash. The fractions were pooled and dialyzed into the aforementioned buffer. The protein was then loaded onto a heparin−agarose column and eluted using a 0 to 200 mM KCl gradient. Fractions containing AbrB were pooled and dialyzed into 10 mM Tris-HCl (pH 8.3 at 4 °C or pH 7.9 at room temperature), 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM KCl, and 10 mM βME. The AbrB was then further purified using a Sephacryl S-100 26/60 column. The fractions containing pure protein were pooled, concentrated, and again dialyzed into 10 mM Tris-HCl (pH 8.3 at 4 °C or pH 7.9 at room temperature), 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM KCl, and 10 mM βME. Throughout the protocol, the presence of AbrB was monitored by 12% tricine gel electrophoresis.

Proteins and DNA. Apomyoglobin (MW ~ 16 950 Da) and human electron transferring flavoprotein, hETF [MW ~ 62 000 Da; a noncovalent complex of one α-subunit (27 kDa), one β-subunit (33 kDa), one FAD, and one AMP] were used as reference proteins in the SEC column to compare the elution time with AbrB. Standard horse skeletal muscle apomyoglobin was purchased from Sigma (St. Louis, MO). Recombinant hETF protein was expressed and purified in the laboratory of J. Vockley at the Mayo Clinic (*19*). All incubations were prepared from a stock solution of 25 μM AbrB stored in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM βME, and 50 μg/mL phenylmethanesulfonyl fluoride (PMSF).

The *sinIR* oligonucleotide, 5′-CTA GAT TTA ATG GCA AAT GAC TTC CAG AGA-3′, and the complementary strand were synthesized in the Molecular Biology Core Facility at the Mayo Clinic. Oligonucleotides were annealed in 100 mM NH_4HCO_3 by heating to 100 °C for 10 min and cooling to room temperature.

PsC-SEC−μESI-MS. Buffered AbrB, apomyoglobin, and hETF [10 mM Tris, 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM KCl, 10 mM βME, and 50 μg/mL PMSF (pH 7.8)] were combined in the PsC in a ratio of 1:4:1. The sample was then pneumatically loaded with 10 psi of nitrogen into a 10 μL loop configured on a 10-port valve (Valco Instruments Co. Inc., Houston, TX). The AbrB−*sinIR* protein−DNA complex was formed by incubation of the two individual components at 37 °C for 30 min. The complex was then introduced and incubated in the PsC for 30 min at room temperature along with *sinIR* and AbrB. All components were in 10 mM Tris, 1 mM EDTA, 0.1 mM $MgCl_2$, 10 mM KCl, 10 mM βME, and 50 μg/mL PMSF (pH 7.8). At the appropriate time, the sample was pneumatically loaded onto the sample loop in the multiport valve. Once the loop was filled, a volume of 1−10 μL was loaded onto the SEC column using timed partial loop injections.

Proteins and protein complexes were desalted or buffer exchanged and separated on a Superdex 200 size exclusion column (0.8 mm × 150 mm; LC Packing, Inc., San Francisco, CA) using a mobile phase of 20 mM NH_4HCO_3 in H_2O at a rate of 8−10 μL/min. This procedure buffer exchanges the nonvolatile salts and other detrimental components with a buffer containing a volatile ammonium salt suitable for the electrospray process. A six-port valve (Rheodyne, Rohnert Park, CA) was configured postcolumn to divert the salts away form the mass spectrometer after elution of the complexes and proteins and to flush the postcolumn tubing with mobile phase buffer prior to the next sample. The postcolumn SEC eluent was split to allow a flow of 300−500 nL/min into a low-flow electrospray interface using fused silica emitters (*20*−*22*). All mass spectrometry analyses were performed on a MAT 900 mass spectrometer (Finnigan Corp., Bremen, Germany) of electrostatic analyzer magnet (EB) geometry. MS measurements of protein mixtures were performed in the positive-ion mode. MS measurements of the DNA and protein−DNA interactions were performed in the negative-ion mode. SF_6 was introduced through the auxiliary port at a rate of 1.6 L/min to prevent source corona discharge. The μESI source voltage was set at 3.5 kV with a capillary temperature of 120 °C. The magnet (B) was scanned from *m*/*z* 1000 to 6000 at a rate of 10 s/decade. A position- and time-resolved ion counter was used for ion detection. Multiple scans were summed over the time range of elution and analyzed using the Finnigan MAT software.

RESULTS

Analysis of the AbrB Protein Multimer. As noted above, the functional form of AbrB has been reported to exist as a homohexamer (*8*, *9*). In particular, Klein et al. determined that AbrB from *B. stearothermophilus* was a hexamer using size exclusion chromatography (termed gel filtration chromatography) with UV detection (*9*). The FPLC column that was used was calibrated with a series of molecular mass markers that included dextran blue (~2000000 Da), bovine serum albumin (BSA) (~66 kDa), aldolase (~44 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (~12.4 kDa), and aprotinin (~6.5 kDa). AbrB protein from *B. stearothermophilus* eluted between aldolase and BSA, but closer to BSA. From these data, it appeared that AbrB had a molecular mass of ~64 kDa, which correlated with homohexamer assembly (*9*).

We initially carried out PsC-SEC−MS analysis on the individual proteins, AbrB, human electron transferring flavoprotein (~62 kDa) (*16*), and apomyoglobin (~16.9 kDa) to

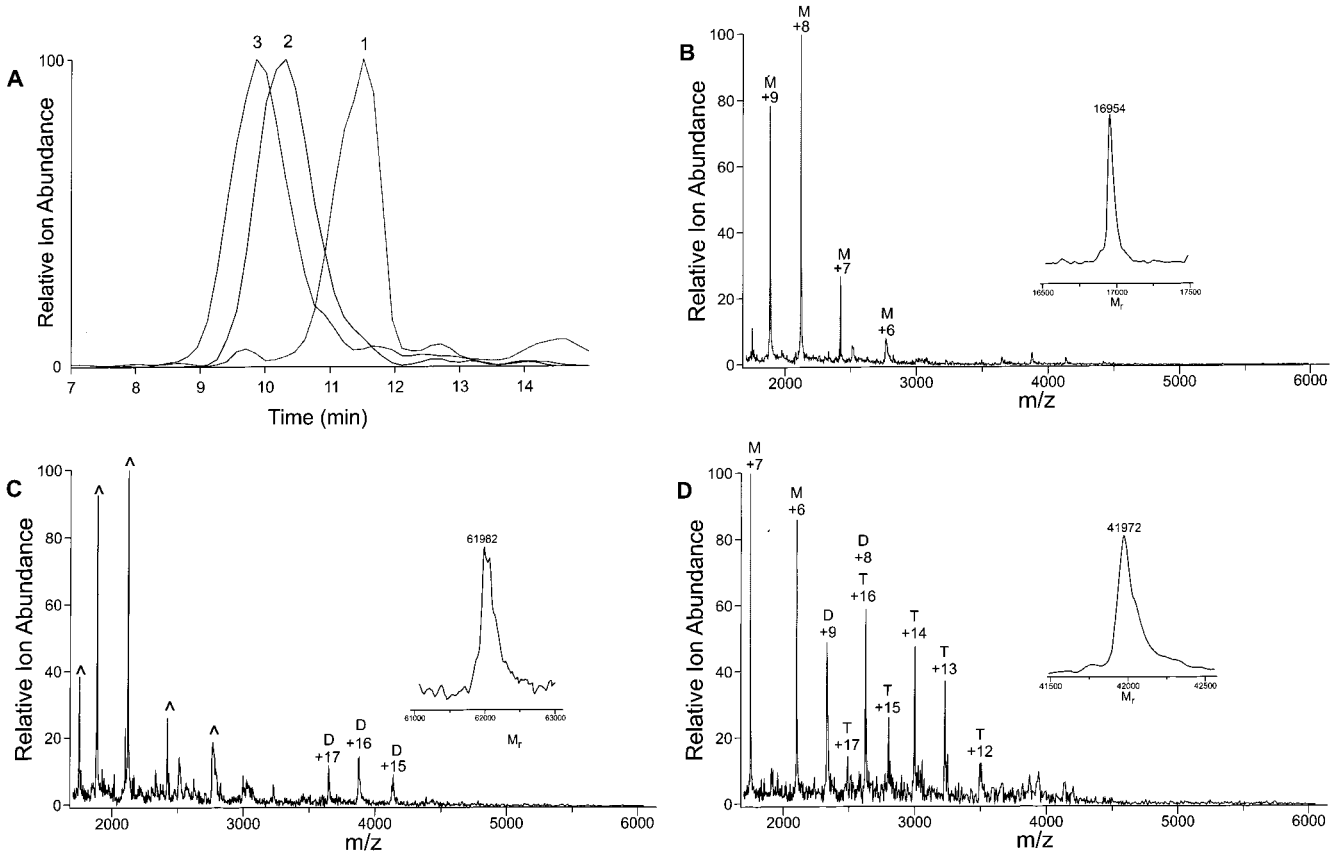

FIGURE 2: PsC-SEC−MS analysis of an ~1:1:4 AbrB/hETF/apomyoglobin mixture. (A) Elution ion chromatogram of the three proteins off the SEC column and analyzed by μESI-MS. On the basis of individual elution times, peak 1 corresponds to apomyoglobin, peak 2 to hETF, and peak 3 to AbrB. (B) Positive-ion μESI mass spectrum of peak 1 from summed scans resolved from peak 2. The multiply charged spectrum is shown over the *m*/*z* range of ~1750−6100 amu. The multiply charged ions labeled "M" correspond to different charge states (e.g., +9 and +8) of the apomyoglobin monomer. Upon transformation of these multiply charged ions, a relative molecular mass (M_r) of 16 954 Da (±0.01%) is observed corresponding to apomyoglobin (inset). (C) Positive-ion μESI mass spectrum of peak 2. The summed scans afford a multiply charged spectrum (~1750−6100 amu) with two distinct ion series present. The ions labeled "∧" correspond to the apomyoglobin monomer. The ions labeled "D" correspond to the heterodimer of hETF. Transformation of the ion series labeled D affords an M_r of 61 982 Da, corresponding to α,β hETF. (D) Positive-ion μESI mass spectrum from all scans from peak 3 that were resolved from peak 2. The multiply charged ion series (~1750−6100 amu) are denoted by M, D, or T, and the charge state (e.g., +6, +9, etc.) is also shown. Ions marked M correspond to monomer AbrB, those marked D to the AbrB homodimer, and those marked T to homotetramer. Transformation of the ion series labeled T reveals an M_r of 41 972 Da (inset), corresponding to the homotetramer of AbrB. The presence of M and D ions is caused by spray conditions (see the text).

determine approximate elution times (data not shown). We subsequently subjected a 1:1:4 molar equivalent mixture of AbrB, hETF, and apomyoglobin to PsC-SEC−MS. In this case, however, the detector was not UV but μESI-MS. The elution ion chromatogram is shown in Figure 2A. Apomyoglobin is the late-eluting protein and is detected at an elution time of ~11 min and 45 s (peak response 1), commensurate with its small hydrodynamic volume (or size). Peak 2 elutes at ~10 min and 25 s and corresponds to hETF. Peak 3, which is AbrB, actually elutes fastest but close to hETF. On the basis of simple elution profiles, one would conclude from these data that the molecular mass of AbrB is >62 kDa and, hence, a homohexamer. On the basis of these data, the conclusion would be in agreement with Klein et al. (*9*). However, as has often been stated, size exclusion chromatography does not accurately predict molecular mass but hydrodynamic molecular volume (*15*).

Inspection of the PsC-SEC−MS data for the three proteins indicates that a different conclusion with regard to the nature of the multimer form of AbrB is in order. Transformation of the multiply charged μESI spectrum for apomyoglobin (Figure 2B) affords an M_r of 16 954 Da (±0.01%) (inset of Figure 2B). The multiply charged spectrum for hETF reveals two sets of multiply charged ions (Figure 2C). The ion series labeled "∧" is due to the presence of apomyoglobin also in this elution volume. The ion series between *m*/*z* ~3700 and 4400 labeled "D" on transformation affords an M_r of 61 982 Da (±0.01%) corresponding to the $\alpha\beta$ heterodimer hETF. It is of interest to note that the α subunit contains a single molecule of noncovalently bound ATP and the β subunit contains noncovalently bound FAD (*16*). Both ligands remain associated with the protein and are detected as part of the protein−protein−ligand complex. However, the multiply charged ion series for AbrB reveals an ion series between *m*/*z* ~2500 and 3500 labeled "T" (Figure 2D). This ion series, on transformation, reveals an M_r of 41 972 Da corresponding to the homotetramer of the protein (see the inset of Figure 2D). The ion series labeled "M" and "D" (Figure 2) correspond to the AbrB monomer and homodimer, respectively. These species are formed in the source region of the mass spectrometer. This is brought about by some in-source collision-induced dissociation of the homotetramer, and we have described this phenomenon in detail elsewhere (*17*, *18*). In all the analyses carried out by PsC-SEC−MS of

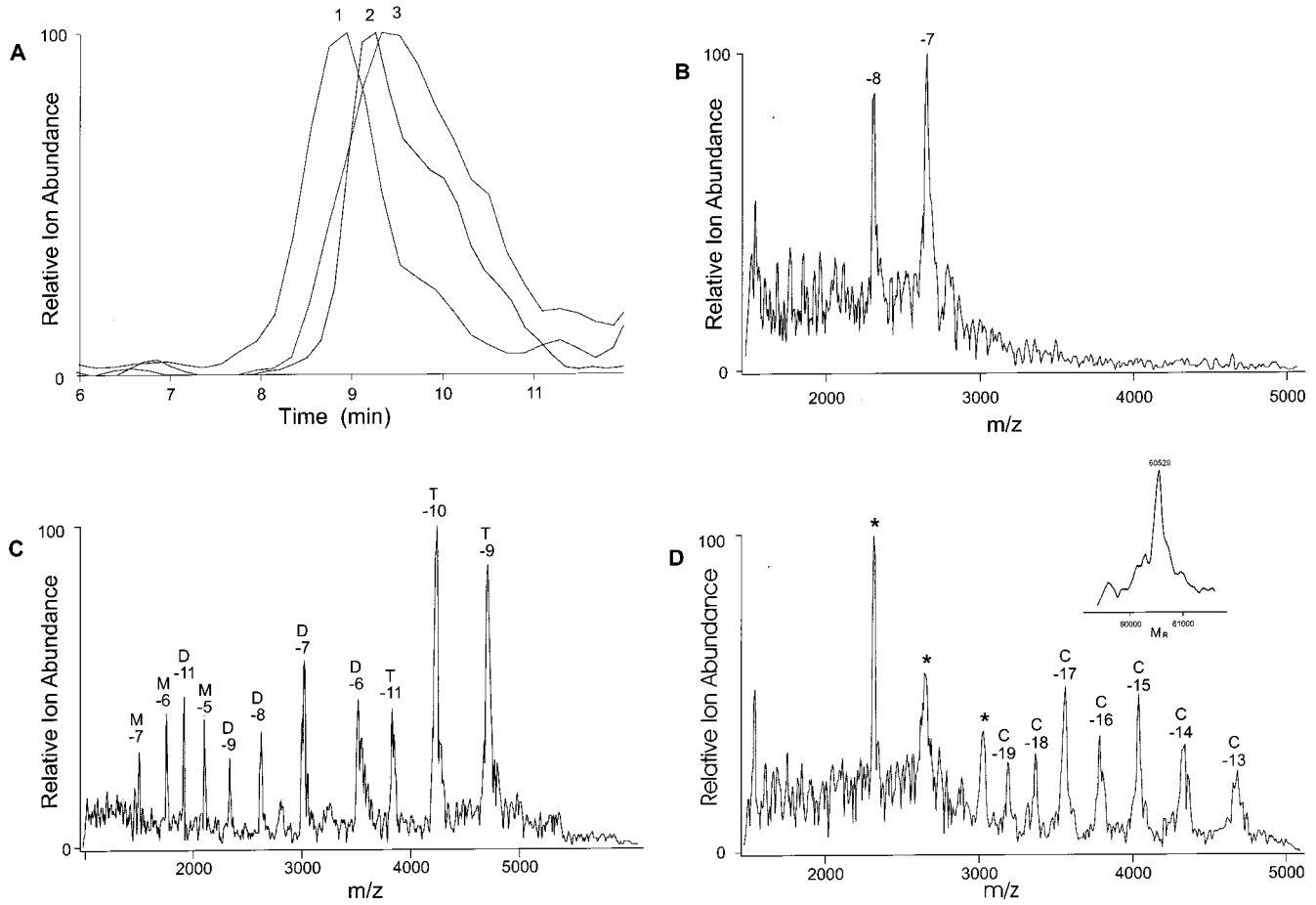

FIGURE 3: PsC-SEC−MS analysis of an ∼1:1:1 mixture of *sinIR* double-stranded DNA, AbrB protein, and the *sinIR*−AbrB complex. (A) Elution ion chromatogram of the *sinIR*/AbrB/*sinIR*−AbrB mixture after coming off the SEC column and analyzed by μESI-MS. Three partially resolved components labeled peaks 1−3 were detected. (B) Negative-ion μESI mass spectrum of peak 1. Only those scans fully resolved from peak 2 were summed to afford the multiply charged spectrum ($m/z \sim 1500-5100$ amu). Transformation of the ion series afforded an M_r of 18 484 Da, corresponding to *sinIR* double-stranded DNA (not shown). (C) Negative-ion μESI mass spectrum of peak 2. The multiply charged ions ($m/z \sim 1000-6000$ amu) contained three distinct series. Ions labeled M correspond to the AbrB monomer, those labeled D to the AbrB homodimer, and those labeled T to the homotetramer. The ion series labeled T on transformation afforded an M_r of 41 971 Da (not shown). (D) Negative-ion μESI mass spectrum of all scans from peak 3 resolved from peak 2. The multiply charged ions ($m/z \sim 1500-5100$ amu) revealed a series labeled C. These ions on transformation afforded an M_r of 60 529 Da, corresponding to the AbrB homotetramer bound to *sinIR* double-stranded DNA (in the inset). The ion series denoted with asterisks correspond to free *sinIR*.

AbrB, there was no detectable evidence for a noncovalent complex at ∼62−64 kDa corresponding to the homohexamer. Hence, one concludes that the hydrodynamic molecular volume of AbrB from SEC chromatographic behavior is comparable to that of BSA or hETF. However, the actual measured M_r of 41 972 Da indicates unequivocally that it is a homotetramer protein complex.

Analysis of AbrB−Target DNA Complexes. Previously, Strauch has reported K_d values for a number of different target DNA sequences with AbrB (*10*) using a gel retardation assay employing end-labeled [α-^{32}P]DNA. One natural promoter, *sinIR*, was determined to bind to AbrB with a K_d of ∼200−1000 nM, depending on DNA length. In this work (*10*), as well as others (*9*), the exact stoichiometry of any AbrB−target DNA complex was not determined. Here, we subjected an ∼1:1:1 molar equivalent mixture of *sinIR*, AbrB, and the *sinIR*−AbrB complex to PsC-SEC−MS analysis.

The ion elution chromatogram afforded three major but partially resolved elution ion profiles (Figure 3A). The negative-ion μESI-MS spectrum for ion chromatogram peak 1 showed a simple ion series between m/z ∼2500 and 2800 (labeled −8 and −7) (Figure 3B). Transformation of this ion series revealed that this corresponds to the double-stranded DNA *sinIR* with an M_r of 18 555 Da (data not shown). The ion elution signal labeled peak 2 revealed in the negative-ion μESI spectrum the presence of a number of ion series (labeled M, D, and T in Figure 3C). On transformation, the ion series labeled T ($m/z \sim 3800-4800$) revealed an M_r of 41 971 Da, identifying this as the homotetramer AbrB. It should be noted that the slight differences in spectral data observed in Figures 3C and 2B for AbrB are that in the former case it is negative-ion and in the latter positive-ion μESI-MS. Inspection of the third peak, labeled 3 (present in Figure 3A), revealed a plethora of negative ions between m/z ∼3200 and 4800 annotated C (Figure 3D). Transformation of this ion series afforded an M_r of 60 529 Da. This corresponds to the *sinIR*−AbrB DNA−protein complex. Furthermore, these data clearly show that AbrB binds not only to *sinIR* but also as the homotetramer in a 4:1 AbrB−*sinIR* complex.

It is extremely interesting to note that the 4:1 AbrB−*sinIR* complex (peak 3) actually elutes later than the AbrB protein (peak 2) alone (Figure 3A). Simple consideration of molecular mass differences would indicate that the 4:1 AbrB−

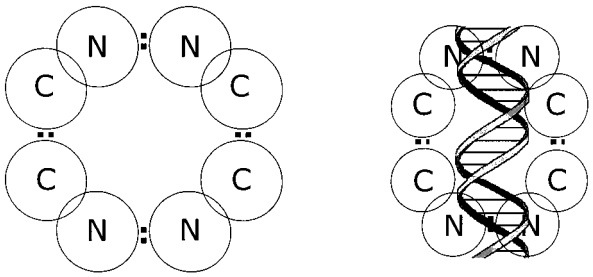

FIGURE 4: Schematic representation of the reduction in hydrodynamic volume as the AbrB homotetramer binds to *sinIR*. On the basis of elution times from SEC and M_r values from μESI-MS, the late-eluting peak 3 which corresponds to a 4:1 AbrB−*sinIR* complex (M_r = 60 529 Da) has a smaller hydrodynamic volume (size) than the homotetramer AbrB alone.

sinIR complex (M_r = 60 529 Da) should elute before the AbrB protein homotetramer (M_r = 41 972 Da). However, as noted above and elsewhere (*15*), SEC separation relies on hydrodynamic volume (or size) and not the molecular masses of components. Therefore, one must also conclude that binding of AbrB to *sinIR* results in a significant reduction in the hydrodynamic molecular volume (or size) of the protein−DNA complex compared to the protein alone. This is represented schematically in Figure 4. As the AbrB binds to the target DNA, a reduction in the molecular diameter of the protein complex must occur.

DISCUSSION

It is well-known that sizable sample losses occur upon sample handling and that this is a cumulative effect (*14*). Consequently, the ability to introduce complex biological mixtures directly into the mass spectrometer without any additional steps is an enormous advantage. A further benefit can be realized if we ensure that any chromatography step to be employed performs two critical but separate functions. First, it removes nonvolatile salt components from the mixture, replacing them with an appropriate amount of volatile salt just before analysis by μESI-MS. Large biomolecules are usually highly charged and may form adducts with soluble inorganic ions. A fraction of the molecules will form weak, electrostatic complexes with Na^+ or K^+ ions in solution, resulting in a distribution in the mass-to-charge ratio for each analyte molecule in the sample. Exchange of alkali metal cations with volatile NH_4^+ ions against an ammonium acetate or bicarbonate solution results in improvements in the sensitivity of subsequent mass spectra. In addition, the chromatographic procedure that separates the components of the mixture should not adversely affect the complex or, at worst, should only slightly perturb noncovalent interactions. The combination of the pseudo cell with size exclusion chromatography provides a simple and straightforward method that satisfies all these stipulations. The pseudo cell allows the mixing and incubation of several species simultaneously without the need for multiple handling steps, and the SEC capillary column separates analytes on the basis of their hydrodynamic molecular volume or size. It is worth making the point that the SEC portion of this methodology is not for determining size; that is the role of the mass spectrometer. The SEC step is used for *separation* based on gentle analyte−gel pore interactions that conserve noncovalent complexes. The bonus of using SEC for this is that it is an excellent method for removing nonvolatile salts and buffers prior to mass spectrometric analysis. The removal of nonvolatile salts prior to mass spectrometric analysis by microdialysis has been proposed (*23*−*25*). However, the use of a rapid on-line cross-linked polyacrylamide matrix for the general desalting procedure, as discussed here, offers notable advantages. Microdialysis approaches are not as successful with smaller sample volumes, and subsequent dilution effects can produce sensitivity losses. While the microdialysis membranes are excellent at removing and exchanging nonvolatile salts (e.g., Na^+, K^+, and Cl^-), they do not perform well for the removal of other, larger, buffer components such as glycerol, DTT, and methanol. The microdialysis membrane is only suited for aqueous use and is not appropriate for those complexes where a small amount of organic is required. The microdialysis membranes also appear, in our hands, to be quite sensitive to pH and the aforementioned organics. These limitations are overcome by the use of SEC for desalting.

In conclusion, the use of PsC-SEC−MS allows the rapid and unequivocal identification of protein−protein and protein−DNA complexes. It also allows accurate determination of stoichiometries of individual constituents of noncovalent complexes. Finally, such an approach also allows a rapid determination of gross conformational changes that might occur on complex formation. Specifically, we were able to rapidly determine that the transition-state regulator protein AbrB is actually a homotetramer and that it binds to a sequence specific target DNA (*sinIR*) as a homotetramer also. In addition, we noted a dramatic reduction of the hydrodynamic molecular volume of the protein−DNA complex relative to that of the homotetrameric protein. This implies that the AbrB−double-stranded DNA complex has undergone a *significant* change in shape compared to the protein alone. Consequently, the use of PsC-SEC−MS allows combination, separation, and subsequent rapid determination of a number of important properties of a protein complex and accordingly should find extensive use in a variety of applications, including, most notably, functional proteomics.

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