Measurement of Macromolecular Binding Using Electrospray Mass Spectrometry. Determination of Dissociation Constants for Oligonucleotide−Serum Albumin Complexes

Michael J. Greig, Hans Gaus, Lendell L. Cummins, Henri Sasmor, and Richard H. Griffey*

Isis Pharmaceuticals, 2280 Faraday Avenue, Carlsbad, California 92008

Received January 26, 1995

Electrospray mass spectrometry (ES-MS) has demonstrated utility for the detection of noncovalent macromolecular associations.[1,2] Complexes have been observed among oligonucleotides, proteins, enzyme−substrate and enzyme−product complexes, and competition among ligands for a receptor.[3−9] Weaker associations among proteins and peptides also have been observed using ES-MS.[10] Though experiments have shown that gas-phase ions retain solution conformations, arguments that these complexes reflect solution structures or equilibrium distributions have been circumstantial.[11]

Levels of individual free and ligated species can be quantitated directly from their relative abundances within the ES-MS ion envelope. For ligands with multiple binding sites having similar affinities, this information can be used to determine directly the dissociation constants (K_D). This situation is common in biological systems, where weak electrostatic and van der Waals contacts govern recognition.[12,13] In this communication, we demonstrate that K_D values for an albumin−oligonucleotide complex can be measured using ES-MS and that the ES-MS K_D values match solution K_D values obtained using capillary electrophoresis (CE). The dependence of the ES-MS K_D on the ionic strength of the buffer suggests that electrostatic forces dominate the albumin−oligonucleotide interaction.

Figure 1a shows the ES mass spectrum of bovine serum albumin (BSA; ICN Pharmaceuticals, Costa Mesa, CA) at pH 7.5. The spectrum was obtained via infusion of BSA (33.5 ng/μL in 30 mM imidazole buffer containing 10 mM NH_4OAc) at 7 μL/min through the electrospray interface of a Hewlett-Packard 5989A quadrupole mass spectrometer in negative ionization mode.[14,15] Under these conditions, BSA generates an envelope of ions (highlighted with a P) between *m/z* 1500 and 2600, corresponding to the $(M - 26H)^{26-}$ through $(M - 41H)^{41-}$ charge states. Deconvolution of these signals yields a mass of 66 497 Da for the BSA.

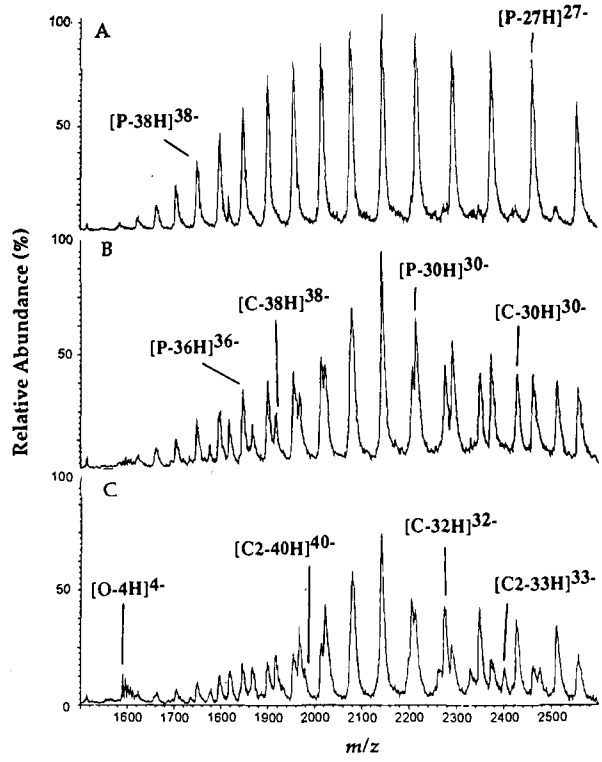

Figure 1. Electrospray mass spectra for (a) 500 nM BSA in 33 mM imidazole−10 mM NH_4OAc buffer, pH 7.5, charge states 26− to 42−; (b) as above with addition of 1.0 μM oligonucleotide; (c) with addition of 3.3 μM oligonucleotide. The signal from BSA is indicated with a P, BSA−oligonucleotide complex with a C, and BSA−oligo^2 complex with a C2.

Mixing BSA with increasing concentrations (0.3−30 μM) of a 20mer phosphorothioate oligonucleotide (GCCCAAGCTGGCATCCGTCA; MW 6367) generates a new series of ions (Figure 1b,c) corresponding to free oligomer (highlighted with an O; *m/z* 1590.9; $(M - 4H)^{4-}$ charge state of oligomer) and the BSA−oligo complex (highlighted with a C).[16] Deconvolution of the new set of signals from the complex yields a mass of 72 870 Da, corresponding to a 1:1 ratio of BSA−oligo. A second complex (MW 79 273; expected MW 79 231), corresponding to a disodium adduct of BSA−oligo^2 (labeled C2), appears at higher concentrations of oligonucleotide. Integrated ion abundances from the various charge states have been summed for BSA and each complex as a function of the concentration of added oligomer. A plot of measured ([P] + [C] + [C2]/[P]) versus added [O] has been fit to a second-order polynomial function.[17] Dissociation constants of 3.1 ± 0.3 (K_D1) and 11.9 ± 0.6 μM (K_D2) have been calculated (R^2 > 0.998) at pH 7.5. Independently, the ratio of K_D1/K_D2 was

(1) Smith, R. D.; Light-Wahl, K. J. *Biol. Mass Spectrom.* **1993**, *22*, 493−501.
(2) Li, Y. T.; Hsieh, Y. L.; Henion, J. D.; Senko, M. W.; McLafferty, F. W.; Ganem, B. *J. Am. Chem. Soc.* **1993**, *115*, 8409−8413.
(3) Griffith, M. C.; Risen, L. M.; Greig, M. J.; Lesnik, E. A.; Sprankle, K. G.; Griffey, R. H.; Kiely, J.; Freier, S. M. *J. Am. Chem. Soc.* **1995**, *117*, 831−832.
(4) Ganem, B.; Li, Y.-T.; Henion. J. D. *J. Am. Chem. Soc.* **1991**, *113*, 7818−7819.
(5) Ganguly, A. K.; Pramanik, B. N.; Tsarbopoulos, A.; Covey, T. R.; Huang, E.; Fuhrman, S. A. *J. Am. Chem. Soc.* **1992**, *114*, 6559−6560.
(6) Baca, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1992**, *114*, 3992−3993.
(7) Ganem, B.; Li, Y.-T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 6294−6296.
(8) Hsieh, Y. L.; Li, Y.-T.; Henion, J. D.; Ganem, B. *Biol. Mass Spectrom.* **1994**, *23*, 272−276.
(9) Jaquinod, M.; Leize, E.; Potier, N.; Albrecht, A. M.; Shanzer, A.; Van Dorsselaer, A. *Tetrahedron Lett.* **1993**, *34*, 2771−2774.
(10) Baczynskyj, L.; Bronson, G. E.; Kubiak, T. M. *Rapid Commun. Mass Spectrum.* **1994**, *8*, 280−286.
(11) Suckau, D.; Shi, Y.; Beu, S. C.; Senko, M. W.; Quinn, J. P.; Wampler, F. M.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 790−793.
(12) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
(13) Misra, V. K.; Hecht, J. L.; Sharp, K. A.; Friedman, R. A.; Honig, B. *J. Mol. Biol.* **1994**, *238*, 264−280.

(14) The N_2 nebulizing gas was maintained at 70 psi, with a 12 L/min flow of N_2 and a 2 L/min flow of O_2 mixed as drying gas at a temperature of 180 °C. Spectra were obtained as the averaged sum of 15 scans from *m/z* 1500 to 2600 at a scan rate of 32 s/scan. The raw data were processed with a 0.5 Da Gaussian filter prior to display. Lens voltages were optimized for the BSA−oligonucleotide complex and did not differ from those used for BSA alone or the BSA−oligo^2 complex.

(15) Imidazole has been used to improve ion abundance, see: Greig, M. J.; Griffey, R. H. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 97−102.
(16) Oligonucleotides were prepared using conventional phosphoramidite chemistry, as described by the manufacturer, on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer. The phosphorothioate oligomer was generated using the Beaucage reagent during oxidation cycles: Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. *J. Org. Chem.* **1990**, *55*, 4693−4698.
(17) Rebek, J., Jr. *Acc. Chem. Res.* **1984**, *17*, 258−264.

0002-7863/95/1517-10765$09.00/0

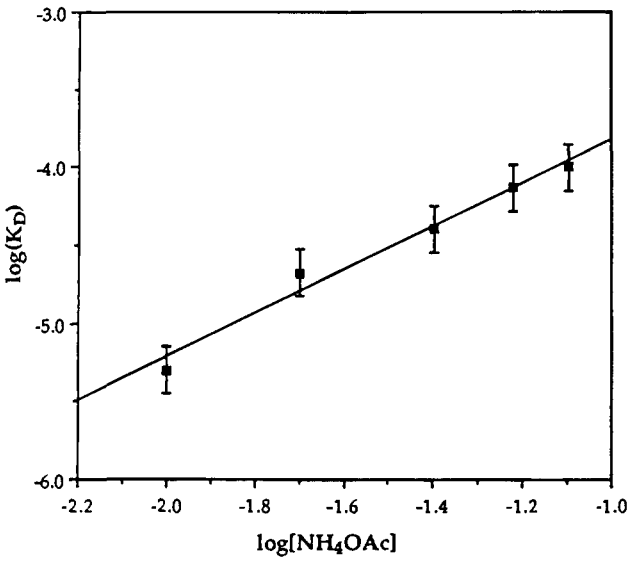

Figure 2. Plot of log(K_D) versus log [NH_4OAc] for the BSA–oligonucleotide complex. Data were obtained by increasing the concentration of NH_4OAc in the buffer while holding the oligonucleotide concentration at 10 mM and the imidazole concentration at 33 mM.

determined to be 0.26 ± 0.01 from direct measurement of [P]-[C2]/[C]^2 for five values of [O].

The value for K_D1 measured using ES-MS is sensitive to the ionic strength of the solution buffer, varying from 0.7 μM in the absence of added buffer to ≥100 μM in the presence of 100 mM ammonium acetate–30 mM imidazole. For an oligo concentration of 10 μM, a plot of log(K_D1) versus log[NH_4-OAc] yields a straight line with a slope of 1.3 and $R^2 = 0.975$ (Figure 2). The K_D1 is sensitive to pH, decreasing to 15 μM at pH 10.0. The dependence of K_D on the ionic strength and pH of the buffer suggests that electrostatic forces contribute significantly to the binding energy of the complex.[18] An analogous peptide nucleic acid (net charge 1+) demonstrated no binding to BSA.[19] However, the structure of BSA also is important, since no detectable binding was observed between heat-denatured BSA and the oligonucleotide. The observed value of K_D also is a function of nucleotide sequence and backbone chemistry.[20]

The dissociation constants determined for this model system with ES-MS have been corroborated using capillary electrophoresis. A 75 μm polyacrylamide-coated column containing 0.1 μM BSA and 25 mM Tris–borate buffer at pH 7.5 was prepared and equilibrated at 25 °C. Differences in oligonucleotide migration time as a function of oligonucleotide concentration were measured following pressure injection. A Scatchard plot of the data produces a biphasic curve. Analysis of the two linear sections generated a K_D2 of 10.0 ± 0.2 μM ($R^2 = 0.97$) for the BSA–oligonucleotide^2 complex, while a K_D1 value of ≤2.8 ± 0.3 μM could be estimated for the BSA–oligo complex ($R^2 = 0.95$).[21,22] The Scatchard analysis of the K_D1 value is an approximation, but it matches the ES-MS K_D1 obtained from a plot of (bound fraction BSA)/[oligo] versus 1/[oligo]. The small differences in K_D values between the two techniques may result from the different buffer required for the CE experiment.

Oligonucleotide antisense therapeutics represent a new of class of drugs. This class of macromolecule has weak interactions with a variety of cellular proteins, but no K_D values have been reported.[23] Results from our attempts to measure dissociation constants between PS oligonucleotides and serum albumins were variable using conventional gel shift, dialysis, or HPLC binding assays. BSA has been used as a model protein to evaluate the utility of ES-MS for determination of dissociation constants and the stoichiometry of oligonucleotide binding. Excellent agreement has been observed between K_D values determined using ES-MS and capillary electrophoresis in this system.

The accuracy of the determination of K_D using ES-MS is governed by several factors. As complete as possible an intergration of the charge envelope for each species has been performed. The most abundant ion shifts from $[M - 31H]^{31-}$ for BSA to $[M - 33H]^{33-}$ for the BSA–oligo complex to $[M - 35H]^{35-}$ for the bis-complex, and in each case, roughly three-fourths of the charge envelope has been observed. These conditions ensure that the relative ion abundances are not affected by instrumental parameters. Decomposition of complexes during transfer from the initial charged droplet to the gas phase would affect the measurement of K_D. However, 2-fold variation in the voltages of the ion optics did not alter relative ion intensities for any species, suggesting that little induced dissociation of the complexes occurs under the conditions described above. The accuracy of the integration process is reduced by the inability to resolve ions where *m/z* values are nearly equal. The charge envelopes are similar in shape for each species, and ion abundances were partitioned by comparison with abundances for neighboring resolved charge states. ES-MS offers a convenient and rapid technique for measuring stoichiometry and dissociation constants for noncovalent macromolecular complexes, where characterization using conventional solution methods proves difficult. Combined ES/FT-MS or ES/MS-MS could be used to characterize the thermodynamics of interactions and to determine the contributions of solvation, electrostatic, and van der Waals interactions toward stabilization of such complexes.

Acknowledgment. We thank S. Crooke for helpful discussions. We also thank L. Meaher for his assistance in optimizing the operation of the mass spectrometer.

Supporting Information Available: Table of *m/z* values for the observed charge states of BSA and the BSA–oligonucleotide complexes (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA950267B

(18) Overman, L. B.; Bujalowski, W.; Lohman, T. M. *Biochemistry* **1988**, *27*, 456–471.

(19) The peptide nucleic acid was prepared and purified following the protocol of Christensen et al.: Christensen, L.; Fitzpatrick, R.; Gildea, B.; Petersen, K. H.; Hansen, H. F.; Koch, T.; Egholm, M.; Buchardt, O.; Nielsen, P. E.; Coull, J.; Berg, R. H. *J. Pept. Sci.* **1995**, *3*, 175–183.

(20) The measured K_D1 for dTGCATCCCCCAGGCCACCAT is 2.1 μM, and a phosphodiester version has a K_D1 of 11.6 μM; data not shown.

(21) Avila, L. Z.; Chu, Y. H.; Blossey, E. C.; Whitesides, G. M. *J. Med. Chem.* **1993**, *36*, 126–133.

(22) Chu, Y. H.; Avila, L. Z.; Biebuyck, H. A.; Whitesides, G. M. *J. Med. Chem.* **1992**, *35*, 2915–2917.

(23) Brown, D. A.; Kang, S. H.; Gryaznov, S. M.; DeDionisio, L.; Heidenreich, O.; Sullivan, S.; Xu, Xiao; Nerenberg, M. I. *J. Biol. Chem.* **1994**, *269*, 26801–26805.