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Conclusive Evidence of the Reconstituted Hexasome Proven by Native Mass Spectrometry

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Supporting Information

ABSTRACT: It has been suggested that the hexasome, in which one of the H2A/H2B dimers is depleted from the canonical nucleosome core particle (NCP), is an essential intermediate during NCP assembly and disassembly, but little structural evidence of this exists. In this study, reconstituted products in a conventional NCP preparation were analyzed by native electrospray ionization mass spectrometry, and it was found that the hexasome, which migrated in a manner almost identical to that of the octasome NCP in native polyacrylamide gel electrophoresis, was produced simultaneously with the octasome NCP. This result might contribute to understanding the assembly and disassembly mechanism of NCPs.

The basic structural unit of chromatin DNA is the nucleosome core particle (NCP), which is essential for compact packaging of DNA in eukaryotic chromosomes. The NCP is composed of a histone octamer [two H2A/H2B dimers and an $(H3/H4)_2$ tetramer] wrapped by approximately 146 bp of DNA, the structure of which has already been determined by X-ray crystallography.[1] It has been suggested that the NCP is constructed by sequential accumulation of histone proteins. First, one $(H3/H4)_2$ tetramer is loaded onto the center of a segment of 146 bp of DNA, and then two H2A/H2B dimers are individually added to organize the residual regions of DNA to form the NCP.[2,3] Disassembly of the NCP is likely conducted in the reverse direction. NCP assembly and disassembly *in vivo* occur with the aid of many proteins, such as histone chaperones and chromatin remodeling factors.[2−6] Meanwhile, the procedure for NCP reconstitution *in vitro* had been established using a histone octamer and a 146 bp DNA fragment in a salt gradient.[1,7,8]

In this study, we tried to prepare the canonical octasome NCP *in vitro* with the conventional procedure using a histone octamer and 146 bp of DNA,[1,8] but we noticed that migration behaviors of the reconstituted products were not uniform in native polyacrylamide gel electrophoresis (PAGE). Because definite identification of the reconstituted products is quite important, we have applied native electrospray ionization mass spectrometry (native ESI-MS), which allows unambiguous mass determination of intact protein assemblies. Furthermore, the stability of the reconstituted NCP was analyzed by native ESI-MS at varying salt concentrations.

The NCP reconstitution *in vitro* was performed with a histone octamer and 146 bp of DNA in a salt gradient from 2 to 0.25 M KCl in the same manner as previously reported.[1,8] Following the removal of nonspecific DNA-histone protein complexes, by heating the sample at 55 °C for 2 h, it was subjected to a continuous-elution electrophoresis cell (Prepcell, Bio-Rad, Hercules, CA) to separate the reconstituted products from free DNA and histone proteins, and the separation process was checked by native PAGE (Figure 1). As shown in Figure 1, free DNA fragments were eluted in advance and completely separated from the high-molecular mass species by electrophoretic purification. Bands for the high-molecular mass species (lanes 7−14), which corresponded to the reconstituted

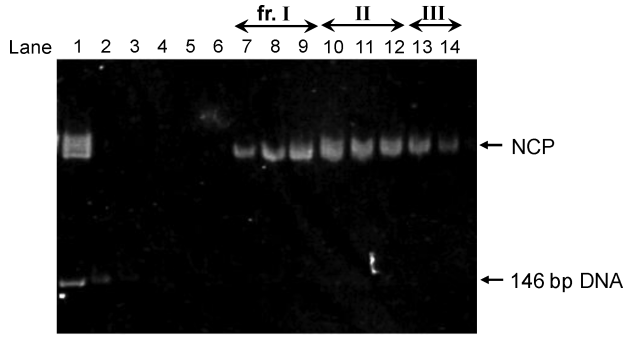

Figure 1. Native PAGE (6% acrylamide) of fractions of the NCP-reconstituted products separated by a continuous-elution electrophoresis cell, Prepcell, and visualized with ethidium bromide. Lane 1 indicates the sample subjected to Prepcell purification. Lanes 2−14 correspond to the eluted samples. Lanes 7−9 (fraction I), 10−12 (fraction II), and 13 and 14 (fraction III) were collected. Lanes 2 and 3 contained the free DNA segment.

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products, showed a gradual shift to a slightly higher position as the elution volume increased. Subsequently, the high-molecular mass species were fractionated into three parts: fractions I (front, lanes 7−9), II (middle, lanes 10−12), and III (back, lanes 13 and 14) (Figure 1).

Fractionated samples were then subjected to native ESI-MS. Because samples for ESI-MS should be prepared in volatile solvents, the fractionated samples were dialyzed against 50 mM ammonium acetate and then applied to native ESI-MS. Figure 2A and Figure S1 of the Supporting Information indicate native

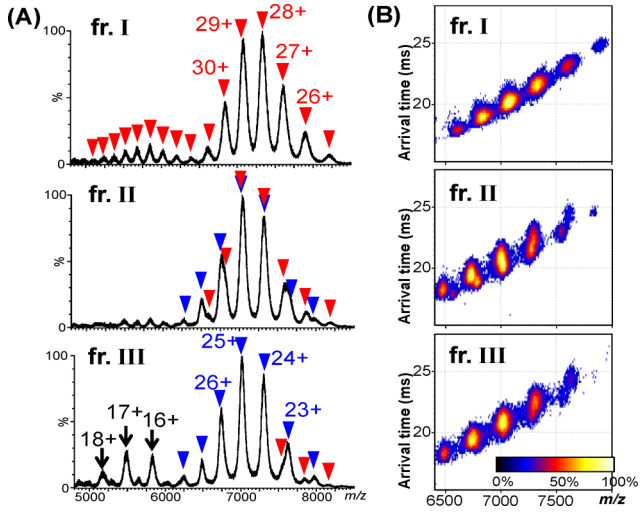

Figure 2. Native ESI mass spectra (A) and IM-MS two-dimensional contour plots (m/z vs arrival times) (B) of reconstituted NCP fractions: (top) fraction I, (middle) fraction II, and (bottom) fraction III. Peaks with red arrowheads in panel A correspond to the multiply charged ions of the octasome NCP; blue arrowheads correspond to the ions of the hexasome NCP, and black arrows correspond to the ions of the 146 bp DNA. Charge states for some observed peaks are indicated above the signals. The color scale of the contour plots is indicated at the bottom of panel B.

ESI mass spectra of reconstituted products (fractions I−III). Mass spectra were obtained by Triwave SYNAPT G2 HDMS (Waters, Milford, MI) with a nanoESI source.[9,10] In the ESI mass spectrum of fraction I, intense signals at m/z 6500−8300 were observed.

These were 25+ to 31+ charged ions of the analyte with a molecular mass of 205177 ± 22, corresponding to the octasome NCP, consisting of a histone octamer and a 146 bp DNA fragment. At m/z 5000−6500, weak signals of more highly charged ions (32+ to 40+) for the octasome NCP were observed. By contrast, in the mass spectrum of fraction III, intense signals at m/z 6400−8100 were observed. These peaks were 22+ to 27+ charged ions of the analyte with a molecular mass of 175304 ± 49, corresponding to the hexasome NCP, consisting of one H2A, one H2B, two H3, two H4, and 146 bp of DNA. In the lower-mass regions, a few signals with relatively weak intensities were observed, which corresponded to the 16+ to 18+ charged ions of free 146 bp DNA. These peaks were almost overlapped with the 30+, 32+, and 34+ charged ions of the hexasome NCP. Because free DNA fragments were removed by electrophoretic separation, the DNA fragments observed in the ESI mass spectrum were likely the dissociation products during MS measurement (further discussed in the Supporting Information). In addition, small peaks at m/z 8162 and 7843 and a shoulder peak at m/z 7590 were assigned to the 25+, 26+, and 27+ charged ions, respectively, of the octasome NCP. In the ESI mass spectrum of fraction II, peaks of multiply charged ions of the octasome and hexasome NCPs were observed; however, signals were overlapped and a little broadened. To verify the existence of the hexasome NCP, these three fractions were also analyzed by ion mobility mass spectrometry (IM-MS), which could resolve multiply charged ions by arrival times as well as m/z values. By IM-MS, ions of the hexasome NCP were clearly recognized in a two-dimensional contour plot of m/z versus arrival time, as shown in Figure 2B and Figure S2 of the Supporting Information.

Subsequently, the stability of the reconstituted NCPs at high salt concentrations was examined by native ESI-MS, as shown in Figure 3. Because it weakens electrostatic interactions, such a

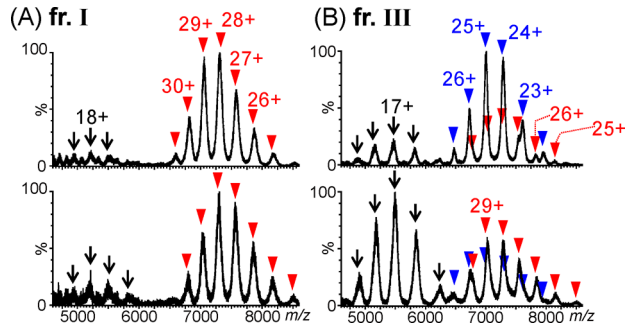

Figure 3. Native ESI mass spectra of the reconstituted NCPs. Fraction I (A) and fraction III (B) were prepared in 0.5 M (top) or 1 M ammonium acetate (bottom). Symbols atop the peaks are as indicated in Figure 2A.

high salt concentration is expected to disrupt histone−DNA interactions, and the reconstituted NCP might be dissociated. In the case of fraction I, the peaks of 25+ through 31+ charged ions of the octasome NCP remained at high intensities in 0.5 and 1 M ammonium acetate. In addition, broad signals with low intensities were observed at m/z 4900−6000, corresponding to 16+ through 19+ charged ions of the 146 bp DNA fragment, and these signals were overlapped with highly charged ions of the octasome NCP.

In contrast, the native ESI mass spectrum of fraction III showed a drastic change in response to the concentration of ammonium acetate (Figure 3B and Figure S3 of the Supporting Information). For the sample in 0.5 M ammonium acetate, little change was observed compared with that in 50 mM ammonium acetate; peaks of the hexasome NCP were observed with high intensities, while 16+ to 19+ ions of the dissociated 146 bp DNA were observed with relatively low intensities (Figure 3B, top panel). At m/z 7826 and 8144, 26+ and 25+ ions of the octasome NCP, respectively, were clearly observed, and these signals were completely separated from the hexasome peaks. For the sample in 1 M ammonium acetate, relative intensities of the hexasome NCP ions were considerably reduced while mainly the peaks of 146 bp DNA were observed in the mass spectrum (Figure 3B, bottom panel). Ions at m/z 6700−8200 were mainly attributed to 30+ through 25+ charged ions of the octasome NCP. In addition, minor peaks were observed at m/z 3200−3700 and ∼2600, corresponding to 15+ through 17+ charged ions for the $(H3/H4)_2$ tetramer and 11+ and 10+ charged ions for the H2A/H2B dimer (Figure S3B of the Supporting Information, bottom panel). This indicated that the

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hexasome NCP easily released the 146 bp DNA segment because it is more unstable than the octasome NCP. Although only a small amount of the octasome NCP was involved in fraction III, the relative intensities of the octasome NCP peaks were improved because the octasome NCP was more stable than the hexasome NCP, even at the high ammonium acetate concentration.

Many cellular functions, such as transcription, replication, and recombination, are highly affected by structural alterations of chromatin. Thus, characterization of intermediate structures of NCP assembly and disassembly processes is key to understanding the activation and repression of these functions. As mentioned above, the NCP can be reconstituted *in vitro* by using a histone octamer and a 146 bp DNA fragment in a salt gradient.[1,7,8] From the suggested mechanism of NCP assembly and disassembly, the hexasome NCP, in which one H2A/H2B dimer is depleted from the canonical NCP, is considered an important intermediate for NCP formation.[11−14] Arimura et al. have successfully reconstituted the hexasome NCP *in vitro*, consisting of a H2A/H2B dimer, a $(H3/H4)_2$ tetramer, and 193 or 112 bp of DNA, and analyzed its biochemical and structural characteristics by micrococcal nuclease digestion and small-angle X-ray scattering, revealing that the hexasome has a globular structure similar to that of the canonical NCP.[15] Unfortunately, no atomic-level structure of the hexasome NCP has been obtained so far, possibly because of the high structural flexibility. To verify the component stoichiometry of the reconstituted NCP, the band intensities of four component histone proteins observed in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) are commonly used. In this study, the fractionated samples were also applied to SDS−PAGE, but it was not easy to differentiate the octasome and hexasome NCPs by SDS−PAGE, as shown in Figure S4 of the Supporting Information. This highlights the significance of native ESI-MS in verification of the reconstituted products before their use in further biochemical experiments.

As described above, this study revealed that the hexasome NCP was formed concomitantly with the octasome NCP in the conventional process of NCP reconstitution with the histone octamer and 146 bp of DNA. Furthermore, the hexasome NCP was rather unstable in the presence of a high salt concentration, with it easily releasing DNA; on the other hand, the octasome NCP in 1 M ammonium acetate was stable enough to survive during native ESI-MS measurements. Because native ESI-MS can determine the molecular mass of even large protein−DNA complexes, this demonstrates conclusive evidence of simultaneous production of the hexasome with the octasome in the NCP assembly process, which might support the hypothesis that NCP assembly and disassembly processes are executed via an intermediate of the hexasome NCP.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting information and methods, Figures S1−S4, and supplementary discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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