Association States of Nucleosome Assembly Protein 1 and Its Complexes with Histones*

Katalin Fejes Tóth‡§, Jacek Mazurkiewicz‡, and Karsten Rippe¶

From the ‡Kirchhoff-Institut für Physik, AG Molekulare Biophysik, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 227 and the §Deutsches Krebsforschungszentrum, Abt. Molekulare Genetik (B060), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

The histone chaperone NAP1 is a carrier of histones during nuclear import, nucleosome assembly, and chromatin remodeling. Analytical ultracentrifugation was used to determine the association states of NAP1 alone and in complexes with core histones. In addition, the concentration dependence of the association was quantified by determining the equilibrium dissociation constant between different NAP1 species. At physiological protein and salt concentrations the prevalent species were the NAP1 dimer and octamer. These were also the association states found to interact with histones in a stoichiometry of one NAP1 monomer per histone. Based on these results a model for a cell cycle-dependent shift of the NAP1 dimer-octamer equilibrium is proposed that reflects different biological functions of NAP1.

In eukaryotes the DNA is packed into nucleosomes consisting of 147 bp of DNA wrapped around an octamer of small basic histone proteins (1–4). This octamer consists of two copies of histones H2A, H2B, H3, and H4, respectively. The nucleosome constitutes the basic repeating unit of chromatin, and formation of a regularly spaced nucleosome chain during chromatin assembly is a prerequisite to maintain the biological activity of chromatin. This requires ATP-dependent chromatin assembly factors as well as so-called histone chaperones (5–10). Histone chaperones bind histone proteins and prevent non-specific, charge-based interaction with nucleic acids. This activity appears essential to cell viability, because simple mixing of histones and DNA in vitro at physiological salt concentrations leads to the rapid formation of poorly defined insoluble aggregates (11, 12). One of these chaperones, the nucleosome assembly protein 1 (NAP1)1 (13, 14), is involved in the transport of the histone H2A-H2B dimer from the cytoplasm to the nucleus and the deposition of histones onto the DNA as described in several reviews (5–7, 10). NAP1 is highly acidic, which is likely to mediate its interaction with the positively charged histone proteins. It is thought to function in large chromosomal domains rather than at local restricted sites (5), and in yeast loss of NAP1 leads to an altered gene expression of about 10% of the genome (15). NAP1 is present at micromolar concentrations throughout the cell cycle, and an increase of the NAP1 concentration during S-phase has been reported for some NAP1 homologues in higher eukaryotes (16–19). Various lines of evidence indicate that NAP1 and the related NAP2 protein are mainly cytoplasmic during G1 and G2 phase with only a small fraction in the nucleus and translocate into the nucleus during S phase (14, 19–21).

In vivo experiments show communoprecipitation of NAP1 with the histones H2A and H2B but not with H3 and H4 (11, 22). In contrast, NAP1 is capable of binding all four core histones in vitro (13, 14, 23–25). Several studies focused on the determination of the association state of NAP1, which in yeast has a monomer molecular mass of 47.9 kDa. By gel filtration or gradient centrifugation very different molecular masses ranging from 120 to 600 kDa and sedimentation coefficients from 1.5 S up to 7 S were reported. In a recent study it was concluded based on analytical ultracentrifugation experiments that yNAP1 exists as complex mixture of species with s values between 4.5 and 12 S at physiological salt concentrations (75 and 150 mM NaCl). In the presence of 500 mM NaCl concentration a 4.5 S NAP1 dimer was identified (26). It appears that upon mixing NAP1 with histones distinct complexes are formed, which sediment around 5–6 S, 8 S, and 10–12 S, respectively (13, 23, 24). Within the 12 S complex all four histones cosedimented in nearly equal amounts, and upon addition of DNA nucleosomes were formed. The 5–6 S complex was assigned to a NAP1 complex with the H2A-H2B dimer and the 8 S complex to a complex that contains NAP1 and the (H3-H4)2 tetramer (13, 23, 24). Hence, the biologically highly relevant interaction of NAP1 with histones appears to be tightly connected to the NAP1 association state. In particular, the nature of the different NAP1 complexes observed previously at physiological ionic strength and their relation to specific NAP1 activities remain to be elucidated.

Here, we have used analytical ultracentrifugation (AUC) to determine the different association states of NAP1 on its own and in complexes with histones. AUC is very useful for the identification of the oligomeric state and the stoichiometry of proteins, and to characterize their thermodynamic and hydrodynamic properties in solution (27, 28). The concentration dependence of the equilibrium between different NAP1 species was quantified in terms of the corresponding dissociation constants. At physiological protein and salt concentrations the dominating species were the NAP1 dimer and octamer, which were also the association states found to interact with histones. Based on these results and estimates of the intracellular concentration of NAP1 we propose a model, in which the transport/exchange of H2A-H2B dimers in G1 and G2 phase is mediated by NAP1 dimers. The accumulation of NAP1 in the nucleus during S phase induces the association of NAP1 into an octamer complex. This species has eight histone binding sites so that it could act as a carrier for multiple histone dimers as required for the de novo assembly of nucleosomes.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 49-6221-54-9270; Fax: 49-6221-54-9112; E-mail: Karsten.Rippe@kip.uni-heidelberg.de
1 The abbreviations used are: NAP1, nucleosome assembly protein 1; AUC, analytical ultracentrifugation; s, sedimentation coefficient; D, diffusion coefficient.

This paper is available online at http://www.jbc.org
Analytical Ultracentrifugation of NAP1 Complexes

EXPERIMENTAL PROCEDURES

Expression and Purification of NAP1—Yeast NAP1 was expressed from plasmid pET28a-NAP1 from Toshio Tsukiyama with a N-terminal His tag. The plasmid corresponds to Saccharomyces cerevisiae NAP1 with a 77 tag cloned into the NdeI site of pET28a. The His tag is followed by a 6xHis cleavage site. NAP1 was expressed with buffer A containing a calculated molecular mass of 52.4 kDa with the His tag and 49.5 kDa after thrombin cleavage. Protein overexpression from pET28a-NAP1 was in Esherichia coli BL21(DE3) carrying the pLYS plasmid. For purification, cells were suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 20 mM imidazole, 5 mM mercaptoethanol, 15% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM Pefabloc), sonicated, and centrifuged. Nickel-chelate beads were equilibrated with the buffer and added to the supernatant after supplementing MgCl2 to a final concentration of 2 mM. Binding was allowed for 3–4 h at 4 °C, and beads were washed twice with lysis buffer and twice with washing buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM imidazole, 5 mM mercaptoethanol, 15% glycerol, 0.01% Nonidet P-40, 1 mM Pefabloc). Elution was conducted with washing buffer supplemented with 0.5 M imidazole. The eluate was dialyzed against buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA). The protein was further purified on a Mono-Q column and eluted with a KCl gradient from 0.1 to 1 M in buffer A. Fractions were checked on SDS-PAGE, and NAP1-containing fractions were pooled and dialyzed against buffer A. DNase activity was tested by incubating NAP1 with supercoiled plasmid DNA over 2 days. For some experiments the His tag was removed by binding purified NAP1 to nickel-agarose and incubation with biotin-tagged thrombin for 2 h at 0 °C using the thrombin tag was removed by binding purified NAP1 to nickel-agarose and incubation with biotin-tagged thrombin for 2 h at 20 °C using the thrombin cleavage kit according to the specifications given by the manufacturer (Novagen, Madison, WI). Thrombin was subsequently removed with streptavidin beads. Using the program SEDNTERP (29) the monomer extinction coefficient of NAP1 was calculated from the amino acid sequence to be ε280 = 57000 M⁻¹ cm⁻¹. From the absorbance spectrum of the protein we derived the corresponding value of ε280 = 293,000 M⁻¹ cm⁻¹.

Preparation and Gel Electrophoresis Analysis of Fluorescent Histone Complexes—Recombinant Xenopus laevis histones H2A12C, H2B, H3C110A, and H4K5C were overexpressed from pET plasmids in E. coli as described previously (30, 31). The H2A12C and H4K5C proteins contain a cysteine substitution, allowing specific labeling with thiol-reactive probes (32, 33). In Histone H3C110A the cysteine is substituted against alanine to hinder modification of this residue, which could lead to functional impairment. Histones H2A12C and H2B or H3C110A and H4K5C were dissolved in unfolding buffer containing 7 M guanidinium chloride, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 5 mM mercaptoethanol. The absorbance values were above 13.3 McM⁻¹ cm⁻¹. Refolding was by dialysis into the same buffer but without guanidinium chloride. A reducing agent was removed by repeated concentration and dilution of histone proteins in freshly degassed labeling buffer (20 mM Tris-HCl, pH 7.2, 0.1 mM EDTA) using Vivaspin 20 concentrators (Vivascience, Hannover, Germany). Labeling was carried out at a histone monomer concentration of ~60 µM with a 1.5 times molar excess of Alexa Fluor 488 C5 maleimide (Molecular Probes Europe BV, Leiden, Netherlands) for 1 h. The reaction was stopped by adding 10 mM dithiothreitol and incubating for 30 min on ice. Labeled histone complexes were then purified from misfolded histone and free dye using BioRex 70 resin (Bio-Rad Laboratories GmbH) as described before (32). Concentration of labeled histones and labeling efficiency were assessed by absorbance spectroscopy using an extinction coefficient of ε490 = 72000 M⁻¹ cm⁻¹ for the Alexa 488 label (34) and the published values for the histones at a wavelength of 276 nm (30) after subtracting the contribution of Alexa 488 at this wavelength. A labeling efficiency of 70% was routinely reached for H2A12C and 50% for H4K5C, and the labeled histones behaved identically to the wild type in terms of association state. Agarose gel electrophoresis of NAP1 complexes with labeled histones was conducted on 1% agarose gels in 0.1× TBE (10 mM Tris, 10 mM boric acid, 0.22 mM EDTA) supplemented with 10 mM KCl. NAP1 and histones were mixed prior to loading in 10 mM Tris-HCl, pH 7.5, 150 mM KCl and incubated for at least 10 min at room temperature. Bands were visualized by illumination with a UV-light box at 302 nm and detection by using a charged-coupled device camera.

Analytical Ultracentrifugation—Analytical sedimentation equilibrium ultracentrifugation was carried out at 20 °C on a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics

2 J. Philo, D. Hayes, and T. Laue, available at www.jphilo.mailway.com/download.htm.

RESULTS

NAP1 Associates into Dimer, Octamer, and Hexadecamer—Yeast NAP1 was expressed in Esherichia coli and purified by His tag affinity and ion exchange chromatography (Fig. 1A). A contaminating DNase activity present in the eluate from the nickel-chelating resin was removed by a second chromatography step on a Mono-Q ion exchange column. After the final purification step the protein was more than 95% pure and the preparation had no detectable DNase activity as tested by incubation with supercoiled plasmid substrate at 37 °C over several days and analysis by agarose gel electrophoresis (data not shown). For some AUC experiments the His tag of NAP1 was removed by thrombin digestion to test if the tag effects the association state. The His tag was cut off quantitatively under mild conditions with Phe-specific thrombin. The His tag was removed by thrombin digestion to test if the tag effects the association state. The His tag was cut off quantitatively under mild conditions with Phe-specific thrombin. The His tag was removed by thrombin digestion to test if the tag effects the association state. The His tag was cut off quantitatively under mild conditions with Phe-specific thrombin.

Binding of NAP1 to histones was studied with histone H2A-H2B and H3-H4 complexes that were reconstituted from overexpressed and purified recombinant histones (30). The heterodimer/tetramer complexes were prepared with the fluorescent dye Alexa 488 covalently attached to either H2A or to H4 in a site-specific manner (see “Experimental Procedures”). These labeled H2A or H4 histones displayed an additional

http://www.jbc.org/ Downloaded from http://www.jbc.org/ Downloaded from http://www.jbc.org/ Downloaded from http://www.jbc.org/ Downloaded from http://www.jbc.org/ Downloaded from http://www.jbc.org/ Downloaded from
massie staining in comparison to the H2Af and H4f fluorescence signal
ramer. An SDS-gel is shown with visualization of the protein by Coom-
strates that the histone preparations are highly purified and
stone octamer preparation as a reference. The SDS-gel demon-
previously (31). Histones H2Af and H2B are not separated on the gel.

Fig. 1. A, purification of NAP1 protein from E. coli with nickel-
affinity and ion exchange chromatography. Due to its high number
of negative charges the NAP1 protein displays a somewhat reduced mo-
bility on SDS gels with an apparent molecular mass of ~60 kDa. L,
lysat; S, supernatant; P, pellet; E(Ni), eluate after affinity purification
with nickel-agarose beads; E(Q), eluate from Mono-Q ion exchange
column. B, cleavage of His tag from NAP1. NAP1 was bound to nickel-
agarose beads, and thrombin cleavage was conducted for the times
indicated. Increasing amounts of tag-free NAP1 were released to the
supernatant. At extended incubation times unspecific cleavage by
thrombin resulted in faster moving bands. C, gel electrophoretic anal-
ysis of reconstituted and purified H2Af-H2B complexes and a reconstituted hi-
tones H2A und H2B from X. laevis have a very similar mobility under standard electrophoretic conditions and are hardly separated (37).

The association states of NAP1 in absence of histones were
determined by sedimentation equilibrium ultracentrifugation.
Typical data sets are displayed in Fig. 2A and B), and the
results are summarized in Table I. Initial runs at approxi-
mately physiological salt concentrations (100 mM KCl, 2 mM
MgCl2) revealed a complex multispecies equilibrium. To iden-
tify the basic building blocks of the NAP1 multimers the salt
concentration was varied from 10 mM to 1 M KCl. At 10 mM KCl
an excellent fit of the data was obtained over a concentration
range from 0.5 to 13.4 μM NAP1 with a monomer-dimer model
and a monomer molecular mass constrained to 51.3 kDa as
calculated from the amino acid composition (Fig. 2A) according
to Reaction 1.

\[ 2 \text{NAP1} \rightleftharpoons \text{NAP1}_2 \] (Reaction 1)

A dissociation constant of \( K_{d1-2} = 0.2 \times 10^{-6} \) M was determined.
At NAP1 concentrations well above the dissociation constant (measurements at 6.7, 8.1, and 13.4 μM) the data could be described well with a single component fit. A molecular
mass of 104 ± 4 kDa was obtained with the molecular mass being the only fit parameter and using the calculated values for
the partial specific volume \( v = 0.726 \text{ ml} \cdot \text{g}^{-1} \) of NAP1 and a
buffer density of \( \rho = 1.0133 \text{ g ml}^{-1} \) (see “Experimental Proce-
dures”). This result is in excellent agreement with the calculated
value of 102.7 kDa for the NAP1 dimer. Thus, NAP1 forms a dimer in low salt buffer, which is the building block for
the oligomers formed at physiological salt concentrations. This
finding confirms a previous study, in which the NAP1 dimer
was identified as the basic association state (26). Accordingly,
the molecular mass of the NAP1 dimer of 102.7 kDa was used
in further experiments as a fixed parameter to determine the
higher association states at physiological buffer conditions and
in complexes with histones.

At 100 mM KCl concentration the association of NAP1 into
larger complexes was evident from a comparison of the molec-
ular weight averages. For example at ~2 μM protein concentra-
tion average values of 81 ± 4 kDa (10 mM KCl) and 169 ± 9
dkDa (100 mM KCl) were determined when fitting the data to a
single component model. However, in these experiments sys-
tematic deviations from a one or two component model were
observed in the residuals of the fits, indicating a more complex
equilibrium state. A detailed analysis revealed that the data at
100 mM KCl were best described by a dimer-octamer-hexa-
decamer equilibrium (Reaction 2).

\[ 8 \text{NAP1} \rightleftharpoons 2 \text{NAP1}_4 \rightleftharpoons 2 \text{NAP1}_{16} \] (Reaction 2)

Other models that involve the NAP1 dimer as the smallest unit
e.g. formation of tetramer or hexamer) did not lead to good fits.
Fig. 2B shows a representative data set at 100 mM KCl with a
fit to a dimer-octamer-hexadecamer equilibrium and the dimer
molecular mass constrained to 102.7 kDa. Very good fits to this
model were obtained over a concentration range from 0.5 μM to
32 μM NAP1 monomer with data recorded at 250 and 280 nm.
Values for the dissociation constant of \( K_{d2-4} = (5.8 ± 3.9) \times
10^{-18} \text{ M}^2 \) and \( K_{d4-16} = (1.2 ± 0.3) \times 10^{-6} \) M were determined.
The concentration at which the ratio of NAP1 in the dimeric
and octameric state is 1:1 is \((K_{d2-4})^{1/2} = (1.8 ± 0.4) \times 10^{-6} \) M.
At salt concentrations of 150 mM KCl, 100 mM KCl plus 2 mM
MgCl2, or 10 mM KCl plus 2 mM MgCl2 (Table I) the data were
also best described with a dimer-octamer-hexadecamer equilib-
Analytical ultracentrifugation of NAP1. The left panel shows the experimental data and the fitted curves in dependence of the radial position. On the right the residuals of the fits are plotted. Equilibrium centrifugation experiments were carried out at 20 °C and at 5,000, 7,000, and 10,000 rpm as shown in A and B. Representative sedimentation velocity runs conducted at 35,000 rpm (C) and 42,000 rpm (D) are shown with every fifth scan being included in the plot. A and C, NAP1 in buffer containing 10 mM KCl was fitted with a monomer-dimer equilibrium (Reaction 1) with the molecular mass of the monomer fixed at 51.3 kDa. B and D, NAP1 in buffer containing 100 mM KCl fitted with a dimer-octamer-hexadecamer equilibrium (Reaction 2).
Raising the KCl concentration above 0.5 M led to the dissociation and velocity experiments at both 10 mM and 100 mM KCl. At 100 mM KCl a NAP1 octamer complex was present, suggesting a complex dimer-octamer-hexadecamer equilibrium of NAP1 precluded the identification of additional species with bound histones. Therefore, recombinant histone complexes were prepared with the fluorescent dye Alexa 488 covalently attached to either H2A or H4 in a site-specific manner. These labeled histones H2A and H4 allowed a specific detection of NAP1-histone complexes both by absorbance and fluorescence measurements. The initial characterization of complex formation of H2A-H2B and H3-H4 with NAP1 was conducted using electrophoresis mobility shift assays. Increasing amounts of NAP1 were mixed with labeled histone complexes (H2Af/H18528H4f) and analyzed on agarose gels exploiting the H2Af or H4f fluorescence signal (Fig. 3). Free histones migrate into the opposite direction due to their highly positive charge. Accordingly, they were not visible in the gel, except for nonspecific, hardly migrating aggregates in samples, in which an excess of histones was present (Fig. 3, lanes with 0, 0.4, and 0.8 mM NAP1). Upon addition of NAP1 to histones, a specific complex formed and reached saturation at a ratio of approximately one NAP1 monomer per histone monomer a good fit could be obtained with a dimer-octamer-hexadecamer model. NAP1-H2Af/H18528H4f complexes migrated at an apparent identical height, indicative for the formation of complexes of similar size and charge.

NAP1 Dimer and Octamer Form Complexes with H2A-H2B Dimers—The AUC analysis was conducted with absorbance data recorded at 496 nm. Thus, only the species containing H2A-H2B dimer was detected. For the H2A-H2B dimer alone a molecular mass of 29 ± 4 kDa was determined in equilibrium runs, which is in very good agreement with the 28.5 kDa derived from the amino acid sequence. NAP1 and histone dimer were mixed at different molar ratios. Only at a ratio of one NAP1 monomer per histone monomer a good fit could be obtained with a dimer-octamer model for sedimentation equilibrium and velocity experiments at both 10 mM and 100 mM KCl. A NAP1 hexadecamer species with H2AfH4f complexes migrated at an apparent identical height, indicative for the formation of NAP1-histone complexes both by absorbance and fluorescence measurements.

### Table 1

| KCl (mM) | Basic association state | Abbreviation | \(M_e^{10}\) (kDa) | Equilibrium complexes |
|----------|-------------------------|--------------|---------------------|-----------------------|
| 10       | NAP1 monomer            | NAP1         | 51.3\(^b\)          | Monomer-dimer         |
| 100      | NAP1 dimer              | (NAP1)\(_b\) | 102.7              | Dimer-octamer-hexadecamer |
| 10 + MgCl\(_2\) | NAP1 dimer          | (NAP1)\(_b\) | 102.7              | Dimer-octamer-hexadecamer |
| 10/100   | H2A-H2B dimer          | (H2A-H2B)\(_b\) | 28.5\(^d\) | Dimer-octamer |
| 10/100   | NAP1 dimer with H2A-H2B dimer | (NAP1)\(_b\)-H2A\(^a\)-H2B\(^a\) | 131.2              | Dimer-octamer |
| 10/100   | H3-H4 dimer            | (H3-H4)\(^c\) | 27.2\(^e\)          | Dimer-octamer |
| 10/100   | NAP1 dimers with H3-H4 dimer | (NAP1)\(_b\)-(H3-H4)\(_b\) | 129.8              | Dimer-tetramer-octamer |

\(^a\) Molecular masses were fixed in the analysis with equilibrium association models at these values calculated from the amino acid sequence. 
\(^b\) A molecular mass of 103.8 ± 1 kDa demonstrating the formation of a dimer was determined from a one component fit for NAP1 at concentrations of 6.7 μM and higher. 
\(^c\) MgCl\(_2\) was added at a concentration of 2 mM. 
\(^d\) A molecular mass of 27.5 ± 4 kDa was determined for H2A-H2B dimer from sedimentation experiments at 10 mM and 100 mM KCl with a one-component fit. 
\(^e\) Under the conditions of the experiments most of the H3-H4 was present in the dimer state. A molecular mass of 29.7 ± 5 kDa was determined in equilibrium runs at 10 and 100 mM KCl from a one-component fit.
Analytical Ultracentrifugation of NAP1 Complexes

**Table II**

Hydrodynamic parameters of NAP1 complexes

| Species          | \( S \)  | \( k_{Da} \) | \( f/f_o \) | \( D \times 10^{-6} \) | Model fit Reaction |
|------------------|----------|-------------|-------------|----------------|------------------|
| NAP1             | 2.4 ± 0.2| 51.3        | 1.80 ± 0.16 | 4.1 ± 0.4       | 1                |
| (NAP1)\(_2\)     | 4.2 ± 0.2| 102.6       | 1.56 ± 0.07 | 3.8 ± 0.4       | 1, 2             |
| (NAP1)\(_3\)     | 11.4 ± 0.8| 410.4       | 1.52 ± 0.10 | 2.5 ± 0.2       | 2                |
| (NAP1)\(_4\)     | 21.3 ± 0.5| 820.8       | 1.29 ± 0.05 | 2.3 ± 0.1       | 2                |
| (NAP1)\(_8\)-(H2A-H2B)_1 | 5.5 ± 0.7| 131.1       | 1.45 ± 0.16 | 3.8 ± 0.4       | 3                |
| (NAP1)\(_8\)-(H2A-H2B)_2 | 16.6 ± 2.2| 554.4       | 1.21 ± 0.14 | 2.8 ± 0.4       | 3                |
| (NAP1)\(_8\)-(H3-H4)_1 | 5.5 ± 0.7| 129.8       | 1.44 ± 0.16 | 3.8 ± 0.4       | 4                |
| (NAP1)\(_8\)-(H3-H4)_2 | 10.9 ± 1.6| 259.6       | 1.15 ± 0.14 | 3.8 ± 0.5       | 4                |
| (NAP1)\(_8\)-(H3-H4)_4 | 16.6 ± 2.2| 519.2       | 1.19 ± 0.14 | 2.9 ± 0.4       | 4                |

* The sedimentation coefficient at standard conditions (20 °C, H2O) as determined from single and global fits to models described by Reactions 1–4 as indicated using SEDPHAT. Standard deviations were determined from averaging results from single fits.

* Calculated molecular masses are included as a fixed parameter in the analysis of sedimentation velocity runs.

* Ratio of the measured friction coefficient \( f \) to the friction coefficient \( f_o \) of a sphere with the same volume, including hydration.

* Diffusion constants at standard conditions (20 °C, H2O) determined from the friction coefficient (35).

---

The sedimentation coefficient of the (NAP1)\(_2\)-(H2A-H2B)\(_2\) complex was measured to be 5.5 ± 0.7 \( S \) and for the (NAP1)\(_8\)-(H2A-H2B)\(_4\) octamer complex a value of \( s = 16.6 ± 2.2 \) \( S \) was determined (Table II).

**NAP1 Dimer and Octamer Form Complexes with H3-H4**—The analysis of NAP1 complexes in the presence of H3-H4 was also conducted at 496 nm to detect only histone-containing species. For H3-H4\(_4\) alone a molecular mass of 29.7 ± 5 kDa was determined in equilibrium runs at 10 and 100 mM KCl with a good fit to a one component model. This value is very close to the molecular mass of 27.2 kDa calculated for the dimer, and the fit to a monomer-dimer equilibrium indicated that under these experimental conditions only a small fraction (<15%) associates into the (H3-H4\(_4\))\(_2\) tetramer. This is consistent with previous studies that reported the dissociation of (H3-H4\(_4\))\(_2\) tetramer into dimers at low ionic strength and protein concentration in the micromolar range (39, 40). The formation of a stable (H3-H4)\(_4\) tetramer would require higher protein and salt concentrations than those used here (40).

NAP1 and histones were mixed in equimolar amounts and examined by sedimentation equilibrium (Fig. 5A) and velocity centrifugation (Fig. 5B) at 10 mM and 100 mM KCl. All data sets showed a good fit to a dimer-tetramer/octetamer model with a fixed molecular mass of 129.8 kDa for the (NAP1)\(_2\)-(H3-H4\(_4\))\(_1\) dimer complex as described in Reaction 4.

Thus, H3-H4\(_4\) behaves very similar to H2A-H2B in its interaction with NAP1 but in addition to the dimer and octamer complex an association state forms, in which two NAP1 dimers stabilize the (H3-H4\(_4\))\(_2\) tetramer complex. At 10 mM KCl the complex was the prevalent species, and little octamer complex was present, whereas at 100 mM KCl the concentration of the (NAP1)\(_8\)-(H3-H4\(_4\)) complex formed with a dissociation constant of \( K_{d, s} = 2 \times 10^{-7} \) \( M \) and \( K_{d, t} = 3 \times 10^{-6} \) \( M \). Sedimentation coefficients for the (NAP1)\(_2\)-(H3-H4\(_4\)) and (NAP1)\(_8\)-(H3-H4\(_4\)) complexes were found to be equivalent to those of the corresponding complexes with H2A-H2B within the accuracy of the measurement. Accordingly, the values for these association states were averaged resulting in \( n = 5.5 ± 0.7 \) \( S \) (dimer) and \( s = 16.6 ± 2.2 \) \( S \) (octamer). The sedimentation coefficient of the (NAP1)\(_2\)-(H3-H4\(_4\))\(_2\) complex was determined to be 10.9 ± 1.6 \( S \) (Table II).

**Conformations of NAP1 Complexes Are Derived from Hydrodynamic Measurements**—From the sedimentation coefficients measured for the NAP1 complexes the ratio of the friction coefficient \( f \) to that of a sphere with the same volume and friction coefficient \( f_o \) was calculated (Table II). The relatively high \( f/f_o \) ratios indicate that the conformation of NAP1 dimer and octamer differ significantly from a sphere for which \( f/f_o \) would equal one. Upon binding of histones to NAP1 dimer only a small decrease of the friction coefficient ratio was observed from 1.52 ± 0.10 to 1.45 ± 0.16 for the (NAP1)\(_2\)-(H2A-H2B)\(_1\)

---

**Fig. 3.** Gel shift analysis of NAP1 with the H2A-H2B dimer and the (H3-H4)\(_4\) tetramer. A, increasing concentrations of NAP1 were mixed with 1.0 \( \mu M \) H2A-H2B dimer. NAP1 was completely saturated with histones at a ratio of ~1 NAP1 monomer per histone. B, increasing concentrations of NAP1 were mixed with 1.0 \( \mu M \) H3-H4\(_4\) dimer. At a ratio of ~1 NAP1 monomer per histone NAP1 was completely saturated with histones.
and to 1.44 ± 0.16 for the (NAP1)$_2$-(H3-H4)$_1$ species. In contrast the NAP1 octamer complex adopted a more globular shape when associated with histones, because the value of $f/f_\text{o}$ decreased significantly. In analogy to the stacked annular pentamer-decamer structure formed by the histone chaperone nucleoplasmin (9, 10, 41) we propose the model depicted in Fig. 6A. It is consistent with the hydrodynamic parameters derived from the sedimentation velocity analysis, but it is noted also that other arrangements of NAP1 subunits would be compatible with the measured friction coefficient ratios. In our model the NAP1 octamer forms an annular structure, which can stack to form a hexadecamer. The disk-shaped structure of the depicted octamer has a relatively high friction coefficient, and stacking of two octamers would lead to a reduction of the $f/f_\text{o}$ ratio as observed in the sedimentation velocity runs, which yielded values of $f/f_\text{o} = 1.52 ± 0.10$ (octamer) and $f/f_\text{o} = 1.29 ± 0.05$ (hexadecamer) (Table II). Upon histone binding the NAP1 octamer undergoes a transition to a more compact association state as indicated in Fig. 6A by an accompanying decrease of the central cavity.

The friction coefficients and the known molecular weights can be used to calculate the diffusion constant $D$. The values for $D$ in pure water are listed in Table II. From mobility measurements of other proteins like GFP it is estimated that in the cell the corresponding in vivo value would be 3 to 4 times lower due to the higher viscosity in the absence of any topological constraints to the mobility imposed by chromatin or other cellular structures (42).

**NAP1 Octamer Formation Could Be Induced in Vivo during S Phase by NAP1 Accumulation in the Nucleus**—The number of NAP1 monomers in haploid yeast cells has been determined to be around 8070 molecules in microarray experiments (43). During G1 phase NAP1 is mostly excluded from the nucleus (21, 44). A haploid yeast cell has an average cell volume of 32 μm$^3$ (45). Estimating that about half of the cell volume is occupied by organelles like nucleus, vacuole, mitochondria, and the Golgi apparatus that are inaccessible to NAP1 this would correspond to a concentration of $1 \text{ M}$ NAP1 monomer or $0.5 \text{ M}$ NAP1 dimer. From the dissociation constants $K_{d_{2-8}}$ and $K_{d_{8-16}}$ determined in the AUC experiments it can be concluded that during G1 phase NAP1 is present in the cytoplasm mostly as a dimer. As plotted in Fig. 6B the fraction of NAP1 present in the dimer, octamer, and hexadecamer state would be 94:6:0.1%.

During S phase dephosphorylation of NAP1 leads to its accumulation in the nucleus (14, 19) with some increase in the expression level (18) resulting in a significantly higher NAP1 concentration. The yeast nucleus has a total volume of 3.6 μm$^3$ of which not more than 2.9 μm$^3$ are estimated to be accessible for NAP1 (46, 47). Without considering any increase in the expression level a concentration of $4 \text{ μM}$ NAP1 monomer could be reached if 80% of NAP1 would be localized in the nucleus. This would favor the association of NAP1 dimer into octamer and hexadecamer. For a $2 \text{ μM}$ NAP1 dimer concentration the ratio of dimer:octamer:hexadecamer is 50:38:12% (Fig. 6B). Taking into consideration that upon histone binding the

---

**Fig. 4.** Analytical ultracentrifugation of NAP1 complexes with histone H2A$^*$-H2B dimer. Experiments were recorded at 496 nm. The left panel shows the experimental data and the fitted curves in dependence of the normalized radial position. On the right the residuals of the fits are plotted. Runs were fitted to the model given in Reaction 3 with the molecular mass of the (NAP1)$_2$-(H2A-H2B)$_1$ species constrained to 131.2 kDa. Data acquired at 10 mM KCl (A) and 100 mM KCl (B) are shown. C, sedimentation velocity run at 10 mM and a NAP1/histone ratio of 1:1. Only every fifth scan is shown.
hexadecamer was destabilized and not detected in the AUC experiments the fraction of NAP1 in dimeric and octameric complexes would be 53:47%.

**DISCUSSION**

NAP1 mediates various important biological activities like the rearrangement of nucleosomes during transcription, the shuttling of the histone H2A-H2B dimer from the cytoplasm to the nucleus, and the assembly of newly synthesized DNA into chromatin (see Refs. 5–7, 10 for reviews). These diverse functions of NAP1 are likely to differ with respect to the number and type of histones found in complexes with NAP1. An understanding of the different NAP1 association states is therefore an essential prerequisite for any mechanistic studies of NAP1 activities. Considerable efforts have been made to characterize the multimer equilibrium of NAP1 alone and in complexes with histones (13, 14, 23, 25, 26). However, the physiologically relevant association states adopted by NAP1 remained to be elucidated. Here analytical ultracentrifugation was used to identify the complexes of NAP1 alone and with core histones, and to characterize these with respect to their thermodynamic stability and hydrodynamic shape.

The NAP1 monomer was only observed at unphysiologically low ionic strength and associates into a dimer with high affinity if the salt concentration is raised. At physiological ionic strength an equilibrium between NAP1 dimer, octamer, and hexadecamer was present (Table I). The measured sedimentation coefficients of the monomer (2.4 ± 0.2 S) and dimer (4.4 ± 0.2 S) determined in our analysis are in good agreement with the values measured recently in the presence of 500 mM KCl and 1.8 M guanidinium hydrochloride (26). In addition, a NAP1 octamer and hexadecamer were identified here with s values of 11.4 ± 0.8 and 21.3 ± 0.5 (Table II). The data summarized in Tables I and II lead to the model shown in Fig. 6A, in which the NAP1 dimer associates into a disk-shaped annular octamer. The hexadecamer is formed by stacking two octamers on top of each other. This conformation is consistent with the large reduction of the friction coefficient ratio from $f_f / f_o = 1.52 ± 0.10$ (octamer) to $f_f / f_o = 1.29 ± 0.05$ (hexadecamer). Because these conformations are similar to the pentamer-decamer structure formed by the histone chaperone nucleoplasmin (9, 10, 41), they appear the most likely, although other shapes and arrangements of NAP1 subunits would also be compatible with the observed friction coefficient ratios.

The interactions between NAP1 and core histones were investigated first in gel shift experiments, and a stoichiometry of 1:1 for NAP1-histone complexes was measured, which confirms the results reported previously (25). The NAP1-histone complexes have been described to sediment between 5 and 12 S (13, 14, 23, 24). Here, the species that predominantly form under physiological salt and protein concentrations were identified as a NAP1 dimer bound to a histone dimer (5.5 ± 0.7 S) and a NAP1 octamer-histone complex (16.6 ± 2.2 S) (Table II). No significant shifts in the equilibrium between the NAP1 dimer and the NAP1 octamer were observed upon histone binding under these conditions.

Based on AUC results and estimates of the intracellular concentration of NAP1 the model shown in Fig. 7 was devised for the cell cycle-dependent formation of different NAP1 association states. During transcription chromatin regions have to adopt a more open conformation. Removal of one H2A-H2B dimer from the histone octamer seems to be essential for transcription elongation through nucleosomes (48, 49), and NAP1 and other histone chaperones stimulate the binding of transcription factors to chromatin templates (50, 51). In addition, the histone H2A-H2B dimer exchanges more rapidly than the H3-H4 tetramer in vivo (52, 53). It has been shown that NAP1 is present in complexes with SWR1 that catalyzed the exchange of H2A-H2B dimer to a dimer variant in yeast (54). Thus, as shown in Fig. 7, mediating the dissociation and re-binding of single H2A-H2B dimers during transcription in G1 phase is a likely function of NAP1 dimers that could explain its effect on gene expression (15). During DNA replication in S
The concentration of NAP1 at 10 mM KCl is mostly present as a monomer and a dimer. At higher concentration (100 mM KCl), NAP1 is present as a monomer and a dimer. The K_d values for multimerization of complexes with histones are similar to those of free NAP1. However, no hexadecamer was detected in the presence of histones. The NAP1 octamer-histone complex adopts a more compact conformation than the free NAP1 octamer as inferred from the hydrodynamic analysis. H3-H4 behaves essentially as H2A-H2B except for an additional intermediate complex formed predominantly at 10 mM KCl as described in Reaction 4. B, dependence of the three NAP1 species on total NAP1 concentration. At 1 μM NAP1 monomer concentration the protein is mostly present as a dimer with a ratio of dimer:octamer:hexadecamer of 94:6:0.1 (dotted line). In contrast, around 4 μM NAP1 monomer half of the protein is in larger complexes with relative dimer:octamer:hexadecamer fractions of 50:38:12 (dashed line).

Analytical Ultracentrifugation of NAP1 Complexes

Fig. 6. A, model of association states of NAP1. At low salt concentration (10 mM KCl), NAP1 is present as a monomer and a dimer. At higher salt concentration (100 mM) the smallest association state is the NAP1 dimer. This associates further to an octamer and a hexadecamer with the indicated K_d values. The values in parentheses refer to (K_d)^(1/3), the concentration at which the ratio of NAP1 in the dimeric and octameric state is 1:1. The K_d values of multimerization of complexes with histones are similar to those of free NAP1. However, no hexadecamer was detected in the presence of histones. The NAP1 octamer-histone complex adopts a more compact conformation than the free NAP1 octamer as inferred from the hydrodynamic analysis. H3-H4 behaves essentially as H2A-H2B except for an additional intermediate complex formed predominantly at 10 mM KCl as described in Reaction 4. B, dependence of the three NAP1 species on total NAP1 concentration. At 1 μM NAP1 monomer concentration the protein is mostly present as a dimer with a ratio of dimer:octamer:hexadecamer of 94:6:1 (dotted line). In contrast, around 4 μM NAP1 monomer half of the protein is in larger complexes with relative dimer:octamer:hexadecamer fractions of 50:38:12 (dashed line).

Fig. 7. Hypothetical model for the cell cycle-dependent association states of NAP1. During G_1 phase NAP1 is mainly phosphorylated and thus cytoplasmic. Low concentrations of NAP1 present as dimers in the nucleus participate in chromosome rearrangement during transcription regulation and bind free H2A-H2B dimers. For this function a dimer that can carry the H2A-H2B dimer would be sufficient. During transition into S phase NAP1 is depolymerized and accumulates in the nucleus leading to higher concentrations. This induces the association of NAP1 into the octameric state, which is able to carry multiple H2A-H2B dimers and possibly also H3-H4 dimers to the replication sites. At the end of S phase NAP1 gets phosphorylated again, is exported to the cytoplasm, and dissociates to a dimer.

In summary, NAP1 has various important activities like the import of H2A-H2B into the nucleus and the assembly of two H2A-H2B dimers and possibly also H3-H4 into nucleosomes during replication. Furthermore, it interacts with the histone H2A-H2B dimer during transcription and histone exchange. These processes differ with respect to the number of histones involved. Thus, the quantitative description of the association states of NAP1 alone and in complexes with histones presented here provides new insights into the mechanisms by which NAP1 can exert its different biological functions.

Acknowledgments—The support of Peter Lichter is gratefully acknowledged. We thank Felix Repert, Malte Wachsmuth, Lutz Ehrhardt, and Borries Demeler for help and Tamas Fischer for valuable discussions. We are grateful to Karolin Luger, Gernot Laengst, and Tom Owen-Hughes for providing plasmid vectors. The project was supported by the Volkswagen Foundation in the program “Junior Research Groups at German Universities.”

REFERENCES
1. Arents, G., Burlingame, R. W., Wang, B.-C., Love, W. E., and Moudrianakis, E. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10148–10152
2. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. 2009
Association States of Nucleosome Assembly Protein 1 and Its Complexes with Histones
Katalin Fejes Tóth, Jacek Mazurkiewicz and Karsten Rippe

J. Biol. Chem. 2005, 280:15690-15699.
doi: 10.1074/jbc.M413329200 originally published online January 31, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413329200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 20 of which can be accessed free at http://www.jbc.org/content/280/16/15690.full.html#ref-list-1