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Zinc Release from *Xenopus* Transcription Factor IIIA Induced by Chemical Modifications†

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ABSTRACT: *Xenopus* transcription factor IIIA (TFIIIA) contains two tightly bound intrinsic Zn^{2+} ions that are released through treatment with either *p*-(hydroxymercuri)benzenesulfonate (PMPS) or diethyl pyrocarbonate (DEP) as monitored by the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR). The inactivation of TFIIIA by DEP as detected by an in vitro 5S RNA gene transcription assay was correlated with the extent of modification of histidine residues and Zn^{2+} release. Following reaction with PMPS, the 7S particle was dissociated into free TFIIIA and 5S RNA. This dissociation could be correlated with the extent of modification of cysteine residues as well as the Zn^{2+} release. The dissociation of the 7S particle was reversed by the addition of excess thiol reagent. However, the reversibility could be inhibited by EDTA, suggesting that Zn^{2+} was required for the binding of TFIIIA to 5S RNA. In the presence of PMPS- or DEP-modified TFIIIA or Zn^{2+}-depleted TFIIIA, the fluorescence emission maximum of the hydrophobic probe, 8-anilinonaphthalenesulfonate, was blue-shifted by 30 nm, while only less than a 10-nm blue shift was observed in the presence of either the 7S particle or TFIIIA. These results indicate that the two Zn^{2+} ions in TFIIIA are coordinated with the cysteine and histidine residues and are required for maintenance of the proper conformation of TFIIIA.

Xenopus transcription factor IIIA (TFIIIA)[1] is one of several protein factors required for the transcription of 5S RNA genes. It binds to the intragenic control region (ICR) of the 5S RNA genes with a high affinity to promote the initiation of transcription (Engelke et al., 1980; Sakonju et al., 1981) and associates with the 5S RNA transcript in immature oocytes in the form of a 7S nucleoprotein complex (Picard & Wegnez, 1979; Pelham & Brown, 1980). TFIIIA is also a metalloprotein that contains two tightly bound intrinsic Zn^{2+} essential for the binding of TFIIIA to the ICR of the 5S RNA genes (Hanas et al., 1983b). In order to account for the TFIIIA interactions with DNA, a model has been

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[1] Abbreviations: TFIIIA, transcription factor IIIA; ICR, intragenic control region; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)benzenesulfonate; DEP, diethyl pyrocarbonate; ANS, 8-anilino-1-naphthalenesulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; TNG buffer, 10 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 5% glycerol.

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postulated (Miller et al., 1985) that TFIIIA contains nine repeating polypeptide segments, each being a potential Zn^{2+} binding site and being capable of binding to 5.5 base pairs of DNA. Stoichiometry of Zn^{2+} in TFIIIA is an essential factor to validate any model for TFIIIA. The disgreement on the stoichiometry of Zn^{2+} in TFIIIA (Hanas et al., 1983b; Miller et al., 1985) leads us to employ different techniques to reexamine the Zn^{2+} content of this protein.

It is important to understand the structure of TFIIIA-bound Zn^{2+} ions and their coordination chemistry as well as the functions which they would play. Although Zn^{2+} in TFIIIA has been proposed to coordinate with cysteine and histidine residues (Miller et al., 1985; Diakun et al., 1986), direct examination of the role of these residues by chemical modification would help to elucidate the structure/function relationship of intrinsic Zn^{2+} ions in TFIIIA.

The metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) has been used to monitor Zn^{2+} release from metalloenzymes by *p*-(hydroxymercuri)benzenesulfonate (PMPS), which specifically modifies sulfhydryl groups (Hunt et al., 1984, 1985; Giedroc & Coleman, 1986). Also, diethyl pyrocarbonate (DEP) at neutral or slightly acidic pH values has been shown to modify histidine residues with considerable specificity (Melchior & Fahrney, 1970; Miles, 1977). In the present study, we have carried out the chemical modifications of TFIIIA by either PMPS or DEP, utilizing PAR to monitor the Zn^{2+} release from the protein. We have found that the two tightly bound Zn^{2+} ions were released due to the modification of cysteine or histidine residues by either PMPS or DEP concomitant with the loss of TFIIIA binding activities.

Materials and Methods

Materials. PAR was obtained from Eastman Kodak Corp. DEP, PMPS, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 8-anilino-1-naphthalenesulfonate (ANS) were purchased from Sigma Chemical Co. Chelex 100 (200–400 mesh) was from Bio-Rad Laboratories. Stock solutions of Tris-HCl and NaCl were routinely subjected to passage through Chelex 100 to reduce the level of Zn^{2+} contamination. A 5 mM PAR stock solution was prepared by dissolving the solid dye in deionized water and adjusting the pH to 8.8 by NaOH titration.

Isolation of 7S Particle and TFIIIA. The 7S particle from *Xenopus laevis* immature oocytes was purified as previously described (Hanas et al., 1983a). TFIIIA was further isolated from 7S particle by the ammonium sulfate precipitation method (Shang et al., 1988). The protein concentration was determined either by $\epsilon_{280nm}^{1\%} = 0.67$ (Shang et al., 1988) or by the Bradford method (Bradford, 1976). Zn^{2+}-depleted TFIIIA was obtained by incubation of TFIIIA with 5 mM EDTA at 23 °C for 10 min and subsequent dialysis against the desired Zn^{2+}-free buffers.

Reaction of TFIIIA with PMPS. TFIIIA or 7S particle (1–2 μM) in 0.5 mL of TNG buffer (10 mM Tris, pH 7.5, 0.2 M NaCl, and 5% glycerol) containing 0.1 mM PAR was reacted with PMPS by adding a small volume of 4 mM stock solution prepared in deionized water. Zn^{2+} release from TFIIIA was determined indirectly by monitoring the absorbance at 500 nm due to the formation of a Zn^{2+}–PAR_2 complex. The stoichiometry of Zn^{2+} released was calculated by using a molar absorptivity (ϵ) at 500 nm of 6.6×10^4 M^{-1} cm^{-1} (Hunt et al., 1985).

Reaction of TFIIIA with DEP. The concentration of DEP was determined by the absorbance of *N*-carbethoxyimidazole at 230 nm ($\epsilon_{230nm} = 3000$ M^{-1} cm^{-1}) following dilution of DEP into 10 mM imidazole (pH 7.0). Reaction of DEP with TFIIIA was carried out in 100 mM phosphate buffer (pH 7.0) at 23 °C. The extent of inactivation due to modification was determined by measuring the residual in vitro 5S RNA transcription activity of an aliquot removed from the reaction mixture. The conditions for the in vitro transcription assay have previously been described (Shang et al., 1988). The number of histidine residues being modified was estimated from the molar absorptivity for *N*-carbethoxyhistidine, $\epsilon_{240nm} = 3200$ M^{-1} cm^{-1} (Ovadi et al., 1967).

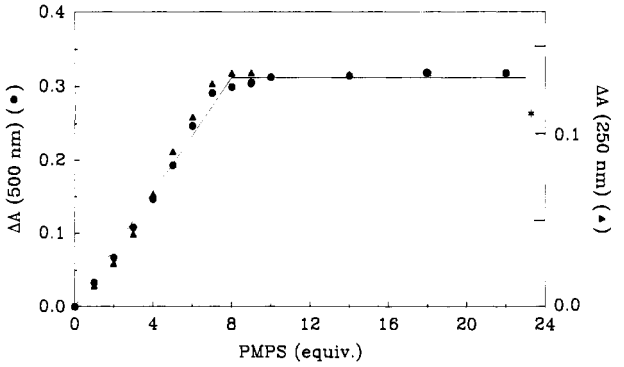

FIGURE 1: Zn^{2+} release and dissociation of 7S particle during the titrations with PMPS. 7S particle (2 μM) in 0.5 mL of TNG buffer containing 0.1 mM PAR was titrated with PMPS at 23 °C. After each addition of PMPS, the absorbance at 500 nm was measured. The spectrophotometer was adjusted to zero absorbance prior to the first addition of PMPS such that the subsequent readings represent ΔA at 500 nm (●). The PMPS titration experiment was also conducted under the same condition except that the absorbance at 250 nm (▲) was monitored and PAR omitted in the solution. The titration curves are shown as ΔA versus the equivalents of PMPS added. The symbol * represents the theoretical change in absorbance if 2 mol of Zn^{2+} are released per mole of 7S particle.

ANS Fluorescence Measurements. The interaction of ANS with TFIIIA was measured in a SPF-500C Perkin-Elmer spectrofluorometer with water-jacketed cuvette holders at 23 °C in TNG buffer containing 25 μM ANS and varying concentrations of TFIIIA. The emission and excitation wavelengths were 530 and 360 nm, respectively.

Zn^{2+} Determination. The Zn^{2+} determinations of the 7S particle and TFIIIA under various conditions were performed with a Perkin-Elmer atomic absorption spectrometer (Model 4500) containing a HGA graphite furnace. All glassware employed in this study was soaked in 30% nitric acid and 10 mM EDTA and rinsed rigorously with deionized water before use. The Zn^{2+} content was calculated from a standard curve constructed by using Zn^{2+} standard solution (Fisher) diluted with deionized water.

Quantitative Gel Analysis. Quantitation of ethidium bromide stained polyacrylamide gel was accomplished by using image analysis hardware from Image Technology, Inc., and software from G. W. Hannaway and Associates (Boulder, CO).

Results

Modification of TFIIIA and 7S Particle with PMPS. The release of Zn^{2+} from the 7S particle during a titration with PMPS was determined by the changes in absorbance at 500 nm in the presence of 0.1 mM PAR (Figure 1). A sequential titration of 7S particle with PMPS resulted in a linear increase in A_{500nm} until a saturation plateau was reached. After the saturation, further additions of PMPS did not give any increase in absorbance. The increase in absorbance at 250 nm, indicative of mercaptide bond formation (Boyer, 1954), was also monitored during the titration with PMPS in the absence of PAR. The good correlation between the titration curves at 500 and 250 nm suggested that Zn^{2+} release from TFIIIA was

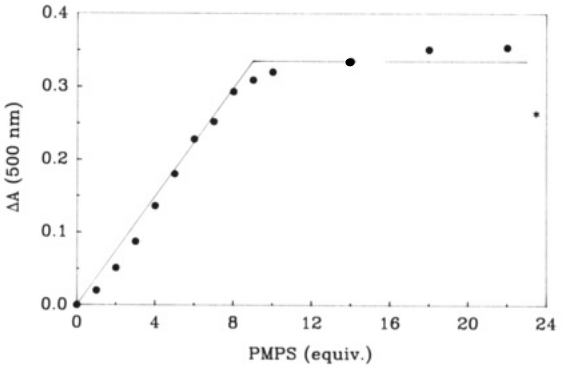

FIGURE 2: Zn^{2+} release from TFIIIA during the titrations with PMPS. TFIIIA (2 μM) in 0.5 mL of TNG buffer containing 0.1 mM PAR was titrated with PMPS as described in Figure 1. Only ΔA at 500 nm was monitored in this experiment.

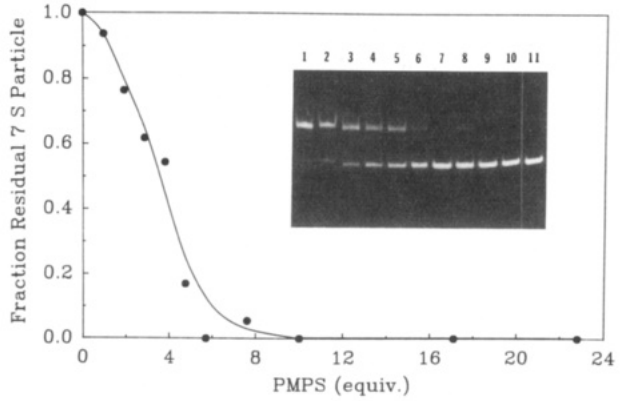

FIGURE 3: Extent of dissociation of the 7S particle as titrated by PMPS. 7S particle (9 μM) in 12 μL of TNG buffer was incubated with various equivalents of PMPS at 23 °C for 5 min and then loaded on a 6% native polyacrylamide gel. The PMPS equivalents used were 0, 0.95, 1.9, 2.85, 3.8, 4.75, 5.7, 7.6, 10, 17.1, and 22.8 for lanes 1–11, respectively (see inset). The gel was stained with ethidium bromide. The bands of 7S particle and 5S RNA on the gel were quantitized as described under Materials and Methods.

due to the modification of cysteine residues involved in the Zn^{2+} coordination. By use of $\epsilon_{500nm} = 6.6 \times 10^4$ M^{-1} cm^{-1} for the Zn^{2+}–PAR_2 complex, the increase in A_{500nm} caused by PMPS was calculated to correspond to the release of 2 mol of Zn^{2+}/mol of protein. Figures 1 and 2 demonstrate that the PMPS titration curves were identical with TFIIIA and 7S particle.

Dissociation of 7S Particle by PMPS. Modification of 7S particle resulted in dissociation of the TFIIIA–5S RNA complex. The extent of the dissociation was monitored by native polyacrylamide gel electrophoresis (inset, Figure 3). Each ethidium bromide stained band on the gel was quantitated by densitometry, and the fraction of residual 7S particle was plotted versus the equivalents of PMPS added. As shown in Figure 3, 8 equiv of PMPS completely dissociated the 7S particle, in agreement with the amount of PMPS needed to release two Zn^{2+} ions from a 7S particle (Figure 1).

Reconstitution of the Dissociated 7S Particle. The 7S particle, dissociated into free TFIIIA and 5S RNA by PMPS modification, could be reconstituted by the addition of excess thiol reagents (10 mM 2-mercaptoethanol or 1 mM DTT). As shown in Figure 4, the 7S particle was completely dissociated by the presence of 10 equiv of PMPS (lane 3), while the reconstitution was accomplished by the addition of either 10 mM 2-mercaptoethanol or 1 mM DTT (lanes 4 and 5). If 5 mM EDTA was added prior to 10 mM 2-mercaptoethanol, the reconstitution of the 7S particle could be prevented (lane

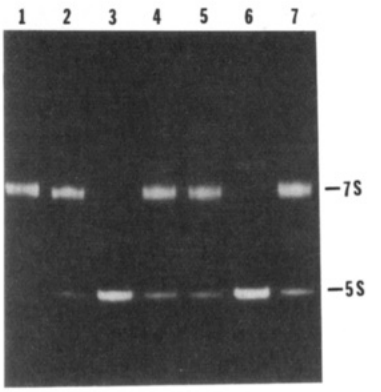

FIGURE 4: Reversibility of 7S particle dissociated by PMPS. 7S particle (7.5 μM) in 10 μL of TNG buffer was reacted with PMPS at 23 °C for 5 min, and the samples were subjected to analysis by 6% native polyacrylamide gel electrophoresis. The amounts of PMPS used were as follows: lane 1, 0 μM; lane 2, 7.5 μM; and lane 3, 75 μM. Following the reaction of 7S particle with PMPS (75 μM), either 1 mM DTT (lane 4) or 10 mM 2-mercaptoethanol (lane 5) was added and incubated for 10 min to reverse the reaction. In lanes 6 and 7, 5 mM EDTA was added before and after the addition of 10 mM 2-mercaptoethanol, respectively.

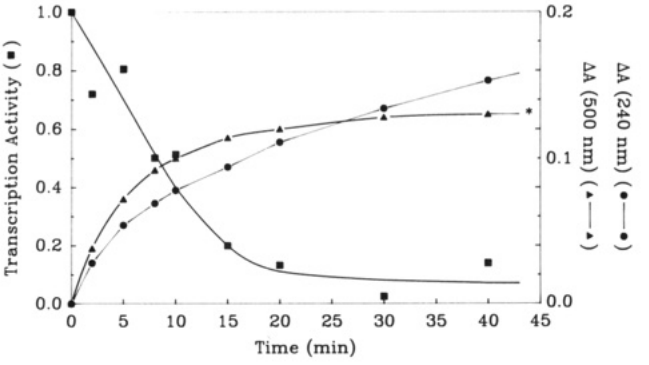

FIGURE 5: Effects of the modification of TFIIIA by DEP. TFIIIA (5 μM) in 10 mM phosphate buffer (pH 7.0) was modified with 180-fold molar excess of DEP at 23 °C, and the change in absorbance at 240 nm was monitored as a function of time (●). At the specified time interval a small aliquot of the reaction mixture was withdrawn for the assay of 5S RNA gene transcriptional activity (■). TFIIIA (1 μM) in 100 mM phosphate buffer (pH 7.0) containing 0.1 mM PAR was incubated with 180-fold molar excess of DEP at 23 °C, and the time-dependent increase in the absorbance at 500 nm was recorded (▲). The symbol * represents the theoretical change in absorbance if 2 mol of Zn^{2+} are released per mole of protein.

6). However, when EDTA was added subsequent to 2-mercaptoethanol, the reconstitution of 7S particle was observed (lane 7), suggesting that Zn^{2+} is essential for the reconstitution.

Zn^{2+} Release and Inactivation of TFIIIA by DEP Modification. Figure 5 shows the time course of Zn^{2+} release from the 7S particle following the addition of a 180-fold molar excess of DEP as monitored by the changes in absorbance at 500 nm in the presence of 0.1 mM PAR in 100 mM phosphate buffer (pH 7.0). On the basis of the extinction coefficient for the Zn^{2+}–PAR_2 complex, the result indicates that approximately 2 mol of Zn^{2+} were released per mole of protein. The incubation of TFIIIA with DEP also resulted in a time-dependent loss in the ability of the protein to stimulate the 5S RNA transcription and a concomitant increase in the absorbance at 240 nm. The reaction of TFIIIA with DEP over a time course of 20 min resulted in approximately seven histidine residues being modified and more than 80% loss in the 5S RNA transcription activity. As shown in Figure 5, there exists a good correlation among the loss of transcriptional activity of TFIIIA, the release of Zn^{2+}, and the modification of histidine residues. The specificity of DEP modification was

Table I: Number of Sulfhydryl Groups in TFIIIA Modified by DTNB[a]

sample	treatment	sulfhydryls/protein (mol/mol)[b]
TFIIIA		7.8 ± 0.8
TFIIIA	5 mM EDTA[c]	7.9 ± 0.7
TFIIIA	5 mM EDTA + 6 M Gdn-HCl[d]	9.5 ± 0.9
TFIIIA	DEP[e]	8.3 ± 1.0

[a] The samples (20 μg of TFIIIA) in 0.5 mL of 100 mM phosphate buffer (pH 7.0) were incubated with 20-fold molar excess of DTNB at 23 °C. The absorbance at 412 nm was recorded, from which the moles of sulfhydryl groups being modified per mole of protein was calculated by using $\epsilon_{412nm} = 1.36 \times 10^4$ M^{-1} cm^{-1} (Means & Feeney, 1971). [b] Average of three measurements. [c] TFIIIA (20 μg) was incubated with 5 mM EDTA in 0.02 mL of 100 mM phosphate buffer (pH 7.0) at 23 °C for 10 min, and the same buffer was then added to give the final volume of 0.5 mL. [d] TFIIIA (20 μg) was incubated with 5 mM EDTA and 6 M Gdn-HCl in 0.04 mL of 100 mM phosphate buffer at 23 °C for 2 h, and the same buffer was then added to give the final volume of 0.5 mL. [e] TFIIIA (20 μg) was modified by 180-fold molar excess of DEP in 0.1 mL of 100 mM phosphate buffer (pH 7.0) at 23 °C for 50 min, and the same buffer was added to give the final volume of 0.5 mL.

checked by ruling out the possible modification of tyrosine, lysine, and cysteine residues in addition to histidine residues. Any decrease in absorbance at 278 nm was not observed, indicating no tyrosine residues were modified by DEP. The loss of transcriptional activity of TFIIIA modified by DEP recovered by the addition of 0.3 M NH_2OH and incubation at 23 °C for 50 min ruled out the possible modification of an essential amino group of TFIIIA by DEP (data not shown). The numbers of cysteine residues accessible to DTNB in the native and DEP-modified protein were not distinct (Table I), indicating that no cysteine residues were modified by DEP.

Quantitation of Sulfhydryl Groups by DTNB. The sulfhydryl specific reagent DTNB was used to quantitate the number of accessible free sulfhydryl groups in TFIIIA (Means & Fenney, 1971). Incubation of TFIIIA with a 20-fold molar excess of DTNB in 100 mM phosphate buffer (pH 7.0) resulted in an increase in the absorbance at 412 nm due to the liberation of thionitrobenzoate anion. Table I shows that approximately 8 sulfhydryl groups were modified in TFIIIA by DTNB under various conditions. TFIIIA treated with 5 mM EDTA and 6 M Gdn-HCl demonstrated no significant increase in the number of sulfhydryl groups being modified.

Conformational Change in TFIIIA upon Removal of Zn^{2+}. Differences in TFIIIA conformation were probed by using the fluorescent hydrophobic reagent ANS (Fairclough & Canter, 1978). Figure 6 shows that, upon addition of Zn^{2+}-depleted TFIIIA to 26 μM ANS in TNG buffer, a shift of the fluorescence emission maximum from 538 to 495 nm occurred concomitant with an increase in ANS fluorescence intensity (curve c). The addition of either TFIIIA or 7S particle resulted in a 10-nm blue shift and a small increase in fluorescence intensity (curve b). The addition of 5 mM EDTA to TFIIIA produced a similar blue shift and fluorescence intensity increase as did the addition to Zn^{2+}-depleted TFIIIA. Both PMPS- and DEP-modified TFIIIA also demonstrated a 30-nm blue shift and a large fluorescence intensity increase (curve c). These results suggest that Zn^{2+} ions coordinated with cysteine and histidine residues are necessary for TFIIIA to maintain its proper conformation.

Zn^{2+} Content in the 7S Particle and TFIIIA. Table II gives the Zn^{2+} content of 7S particle and TFIIIA under various conditions. The 7S particle and TFIIIA contain 2 mol of Zn^{2+}/mol of protein. TFIIIA treated with 5 mM EDTA contains no Zn^{2+}. Interestingly, TFIIIA modified by either

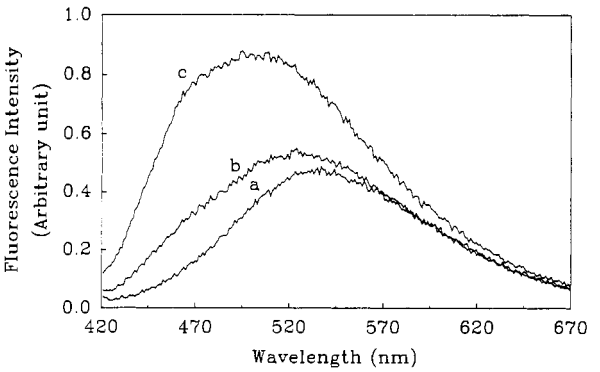

FIGURE 6: Effects of TFIIIA and apo-TFIIIA on the ANS fluorescence spectra. TFIIIA (1 μM) (curve b) or apo-TFIIIA (curve c) was added to ANS (26 μM) in TNG buffer (150 μL). Curve a was the ANS fluorescence spectrum in the absence of TFIIIA. The wavelength of excitation was 360 nm.

Table II: Zn^{2+} Content of 7S Particle and TFIIIA under Various Treatments[a]

sample	treatment[b]	Zn^{2+}/protein (mol/mol)[c]
7S particle		2.21 ± 0.04
7S particle[d]		2.16 ± 0.04
TFIIIA		1.96 ± 0.06
TFIIIA	5 mM EDTA	0.10 ± 0.06
TFIIIA	PMPS	1.88 ± 0.06
TFIIIA	DEP	1.90 ± 0.04
TFIIIA	PMPS + DEP	0.28 ± 0.03

[a] Samples of 7S particle or TFIIIA in TNG buffer were treated with various reagents as indicated below and then dialyzed against the same buffer for 6 h at 4 °C with one change of the buffer. Zn^{2+} content was determined as described under Materials and Methods. [b] For EDTA treatment, the sample was incubated with 5 mM EDTA at 23 °C for 10 min, followed by dialysis. For PMPS treatment, the sample was incubated with 20-fold molar excess of PMPS at 23 °C for 5 min, followed by dialysis. For DEP treatment, the sample was incubated with 180-fold molar excess of DEP at 23 °C for 50 min, followed by dialysis. For PMPS plus DEP treatment, the sample was treated first with PMPS, followed by dialysis, and DEP was then added and the sample was dialyzed again as described above. [c] Average of five determinations. [d] No EDTA was used throughout the purification procedure.

PMPS or DEP also contains two Zn^{2+} per protein molecule, while TFIIIA modified with both PMPS and DEP contains no Zn^{2+}. These results demonstrate that while modification of TFIIIA with either PMPS or DEP only may lower the affinity of the protein for Zn^{2+}, modifications of both cysteine and histidine residues are required for the complete release of Zn^{2+} from TFIIIA.

Discussion

Titration of the 7S particle with 8 equiv of PMPS resulted in the release of two Zn^{2+} ions per protein molecule as monitored by employing the metallochromic indicator PAR (Figure 1). The correlation of this titration curve with the mercaptide bond formation measured at 250 nm implies that the Zn^{2+} release is a result of the modification of cysteine residues which coordinate with the metal ions. Furthermore, the extent of 7S particle dissociation caused by the PMPS modification (Figure 3) also correlated well with the Zn^{2+} release and mercaptide bond formation, suggesting that the Zn^{2+} coordination with cysteine residues is involved directly or indirectly in the binding of TFIIIA to 5S RNA. The PMPS modification of TFIIIA was equivalent to that of the 7S particle under all conditions studied implies that 5S RNA does not interact with TFIIIA in such a way as to inhibit cysteine reactivity. However, it has been observed that 5S RNA in the 7S particle could prevent Zn^{2+} from being chelated by EDTA (see lanes 6 and 7 in Figure 4). One possible inter-

pretation is that although one or more cysteine residues coordinated with Zn^{2+} may be located on the surface of the 7S particle and therefore accessible to PMPS modification, the Zn^{2+} itself is buried within the 7S particle and inaccessible to EDTA. Although there are 23 cysteine residues present in TFIIIA (Ginsberg et al., 1984), only 8 of them could be modified by DTNB. This result is consistent with the requirement of 8 equiv of PMPS for the release of two Zn^{2+} ions per protein molecule and complete dissociation of the 7S particle (Figures 1 and 3). Since the modification of TFIIIA by DTNB also released two Zn^{2+} ions per protein molecule (data not shown), the cysteine reactivity profiles for the DTNB and PMPS modifications are probably identical. Neither the native Zn^{2+}-depleted TFIIIA nor the guanidine hydrochloride denatured protein demonstrated any increase in cysteine reactivity toward DTNB or PMPS, a result indicating that perhaps the inaccessible cysteine residues are buried within the protein or form disulfide bonds. We used excess iodoacetamide to modify the protein in the presence and absence of DTT and separated the modified proteins by acid–urea–polyacrylamide gel electrophoresis (Creighton, 1980). Our preliminary result showed that the protein modified in the presence and absence of DTT had different mobilities, which suggests the existence of disulfide bond(s) in the protein. DEP was used to specifically modify histidine residues in TFIIIA. Seven out of twenty-five histidine residues in TFIIIA modified by a 20-min treatment of DEP led to the release of two Zn^{2+} ions and the loss of transcriptional activity. This result suggests that the two Zn^{2+} ions in TFIIIA are coordinated with histidine residues which are essential for protein function. We failed to estimate how many imidazole groups were modified because of the light scattering of the sample after a 30-min incubation of TFIIIA with DEP.

The dissociation of the 7S particle by PMPS could be efficiently reversed by the addition of excess thiol reagents (lanes 4 and 5 in Figure 3). Two steps are required to reconstitute PMPS-modified TFIIIA into 7S particle. First, Zn^{2+} is reincorporated into the protein. Second, TFIIIA must interact with 5S RNA to form the 7S particle. The high efficiency of reconstitution of 7S particle suggested that perhaps Zn^{2+} was not fully released by cysteine modification, and both cysteine and histidine residues had to be modified before Zn^{2+} was fully released from the protein. This hypothesis was confirmed by the atomic absorption measurements of Zn^{2+} content of TFIIIA treated with either PMPS or DEP, or both (Table II). TFIIIA treated with either PMPS or DEP contained two Zn^{2+} ions while TFIIIA treated with both PMPS and DEP had essentially no Zn^{2+}. Therefore, the two Zn^{2+} ions in TFIIIA were coordinated with cysteine and histidine residues and were only released after modifications of the cysteine and histidine residues by both PMPS and DEP.

The absence of Zn^{2+} release from TFIIIA by PMPS alone described above has raised questions about the quantitation of Zn^{2+} release measured by PAR. If the Zn^{2+} ions modified by PMPS remained coordinated to the protein in the presence of PAR, the expected ratio of 2 PAR molecules to 1 Zn^{2+} ion for the complex formations would actually be 1 PAR molecule for each Zn^{2+} ion. We examined this possibility by incubation of the TFIIIA modified by PMPS with 0.1 mM PAR and dialysis against Zn^{2+}-free buffer. The atomic absorption spectrophotometric analysis revealed that the protein essentially contained no Zn^{2+}, indicating that the modification by PMPS rendered the intrinsic Zn^{2+} ion accessible to chelation by PAR, while PAR did not chelate Zn^{2+} from the protein in the absence of PMPS modification. In other words, the modification by PMPS or DEP alone reduced the affinity of the protein for Zn^{2+} to such an extent that the competing ligand, PAR, could remove the metal. On the basis of the fact that PAR at 10^{-4} M could not chelate out Zn^{2+} from the protein and the affinity of PAR for Zn^{2+}, $K_1' = 4.0 \times 10^6$ and $K_2' = 5.5 \times 10^5$ M^{-1} at pH 7.0 (Hunt et al., 1985), an estimate of the apparent association constant for the Zn^{2+}–protein interaction at pH 7.0 should be greater than 10^{12} M^{-1}.

The fluorescence emission spectrum of ANS was significantly blue-shifted by the addition of the Zn^{2+}-depleted protein or PMPS- or DEP-modified TFIIIA to the solutions containing the hydrophobic fluorophore (Figure 6). These results indicate that the Zn^{2+} domains are probably located in a hydrophobic region of the protein and that more hydrophobic regions of the protein were exposed upon removal of Zn^{2+}. However, attempts to incorporate Zn^{2+} into apoprotein in the presence or absence of ANS, or to reverse the PMPS modification by using excess thiols in the presence of ANS, had not been successful. There are two possible reasons for this. One is that TFIIIA underwent a conformational change upon removal of Zn^{2+} and proper conditions would be needed to overcome a free energy barrier caused by the conformational change in order to restore the Zn^{2+} ions. The other is that ANS prevented the Zn^{2+} ions from incorporating into the PMPS-modified TFIIIA. In the absence of ANS, modification of TFIIIA by PMPS followed by the addition of excess thiols was able to reverse the reaction as monitored by the ANS fluorescence change (from curve c to curve b in Figure 5).

In order to confirm that the two Zn^{2+} ions released by either PMPS or DEP are the two intrinsic to TFIIIA, we performed atomic absorption spectrophotometric analysis on the protein samples under various treatments (Table II). The results showed that either the 7S particle or TFIIIA contains two Zn^{2+} ions per protein molecule, and even the 7S particle which was not exposed to EDTA during the purification procedure contains the same numbers of Zn^{2+} ions. These observations indicate that TFIIIA contains two intrinsic Zn^{2+} ions which can be released by modifications with both PMPS and DEP.

Klug and co-workers (Miller et al., 1985) have proposed that TFIIIA contains nine zinc fingers, a motif whereby one zinc ion is coordinated with two cysteine and two histidine residues. The results of the present chemical modification studies support the chemical aspect of their model. However, unlike our original report (Hanas et al., 1983b) that 7S particle contains two tightly bound Zn^{2+} ions that could not be removed by EDTA, they found that, in the absence of metal chelators, TFIIIA contained 7–11 Zn^{2+} ions (Miller et al., 1985), which was consistent with the number they estimated from the extended X-ray absorption fine structure (EXAFS) studies (Diakun et al., 1986).

We have subsequently used two independent techniques to measure the Zn^{2+} content of TFIIIA. Both the atomic absorption spectrometry and colorimetric measurements gave the same results, consistent with the fact that TFIIIA contains 2 mol of Zn^{2+}/mol of protein (Tables I and II). Analyses of our 7S particle preparations demonstrated that at least 95% of the protein was in the form of 7S particle (e.g., see lane 1 of the inset in Figure 3). Further, the addition of 10 μM exogenous Zn^{2+} ions did not stimulate the binding of our TFIIIA preparations to the 5S RNA gene as assayed by gel retardation experiment using a DNA fragment containing ICR (data not shown). These results suggest that the protein which contains two intrinsic Zn^{2+} ions is highly active, and additional Zn^{2+} ions do not appear to be required to enhance the protein activity with respect to either DNA or 5S RNA binding.

Although we cannot absolutely rule out the possible loss of loosely bound Zn^{2+} during the purification procedure, the presence of these additional Zn^{2+} ions seems not play an important role in the TFIIIA function.

In conclusion, our studies demonstrate that TFIIIA contains two tightly bound Zn^{2+} ions that are coordinated with both cysteine and histidine residues. The modifications of either of these amino acids change the structure of the Zn^{2+} domains and cause the loss of protein activities. These results provide some insight concerning the structure/function relationships of the intrinsic Zn^{2+} ions of TFIIIA.

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Complex Formation between the Adenovirus DNA-Binding Protein and Single-Stranded Poly(rA). Cooperativity and Salt Dependence†

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ABSTRACT: The complex formed between adenovirus DNA-binding protein (AdDBP) and poly(rA) was investigated with circular dichroism spectroscopy. The binding process was studied at a variety of salt concentrations, and the titration curves were analyzed according to the contiguous cooperative binding model given by McGhee and von Hippel [McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489]. The cooperativity factor ω of the binding process is low ($\omega \approx 20$–30) and independent of the salt concentration. This in contrast to the binding constant K for which a moderately strong salt dependence is observed: $\partial \log (K\omega)/\partial \log [NaCl] = -3.1$. The size of the binding site was consistently calculated to be about 13. We also studied the C-terminal 39-kDa fragment which is sufficient for DNA replication in vitro. Complex formation between this fragment of AdDBP and poly(rA) appeared to be characterized by spectroscopic and binding properties similar to those of the intact protein. Only, the binding constant in 50 mM NaCl is a factor of 5 lower.

The adenovirus DNA-binding protein (AdDBP) is an example of the important class of single-stranded DNA binding proteins [see, for reviews, Chase and Williams (1986) and Lohman et al. (1988)]. Apart from a role in control of early (Carter & Blanton, 1978) and late (Klessig & Grodzicker, 1979) transcription and possibly virus assembly (Nicolas et al., 1982), AdDBP is required for DNA replication both in vivo and in vitro (van der Vliet et al., 1975; Kaplan et al., 1979). The protein has a strong stimulating effect on the activity of the adenovirus DNA polymerase on single-stranded templates (Field et al., 1984), probably via a dual mechanism: (1) interaction with the single-stranded DNA, leading to a complex that enhances the rate of polymerization; (2) direct specific interaction with the polymerase. AdDBP binds to ssDNA as well as RNA (Cleghon & Klessig, 1986; Adam & Dreyfuss, 1987; Seiberg et al., 1989). In vitro binding to the attenuated RNA derived from the viral major late promotor has been observed (Seiberg et al., 1989) while also in vitro AdDBP is associated with mRNA and hnRNA (Adam & Dreyfuss, 1987). The physiological meaning of the binding

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