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Restoration of binding of oxidized transcription factor IIIA to 5S RNA by thioredoxin

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ABSTRACT

7S particles from Xenopus oocytes were completely dissociated under non-reducing conditions. Studies using glycerol gradient centrifugation show that unlike the native 7S particle in which 5S RNA and TFIIIA co-sedimented in a fairly sharp peak, the RNA from the denatured 7S sedimented at the position corresponding to the 5S RNA and the TFIIIA sedimented as a wide peak between 6S and 12S. Thioredoxin from E.coli can catalyze the reactivation of the TFIIIA as measured by its ability to reform the 7S particle . The rate of reactivation with thioredoxin was significantly greater than with dithiothreitol. Oxidized thioredoxin was unable to reactivate TFIIIA. Pure TFIIIA can be inactivated and subsequently reactivated in the same way by formation of a cross-linked structure via intermolecular disulfide bridges.

INTRODUCTION

Xenopus laevis factor III A (TFIIIA) is a positive transcription factor that binds to an intragenic control region of the 5S RNA gene , promoting its transcription by RNA polymerase III (1-4). TFIIIA associates with 5S RNA, and in immature oocytes is found in the form of a 7S nucleoprotein complex (5). The ability of TFIIIA to bind either the 5S RNA gene or its product led to the suggestion that it might have a potential role in feedback regulation of 5S gene expression(6). Besides the specific binding to the 5S RNA gene, TFIIIA possesses other multiple activities such as a DNA-dependent ATPase activity (7), the promotion of the reassociation of complementary single stranded DNA (8) and the induction of dynamic chromatin assembly on cloned Xenopus 5S RNA genes (9). TFIIIA is a Zn binding protein (10,11) which contains nine tandem repeats of a 30 amino acid cluster having two cysteines and two histidines in each repeat. This observation led to the suggestion that each repeat binds one atom of Zn.

In this work we present evidence that one possible route of inactivation of TFIIIA, either in pure preparations or as a part of the 7S nucleoprotein particle, is via oxidation and subsequent formation of an heterogeneous

population of molecules crosslinked by disulfides bonds. This inactivation can be reverted by the action of thioredoxin from E.coli.

MATERIALS AND METHODS

Immature Xenopus laevis frogs were obtained from Nasco. Bio Rex 70 and Bio Gel HT were purchased from Bio Rad, and DEAE cellulose from Whatman. Thioredoxin was from Chemical Dynamics Corp.

Purification of the 7S RNP particle

7S RNP particles were isolated using a modification of the procedure of Hanas (12). Briefly, 7S particles isolated by glycerol gradient centrifugation were loaded onto a Bio Rex 70 column equilibrated with 30 mM Hepes, pH 7.5, 0.025 M KCl, 1.5 mM $MgCl_2$, 0,5 mM DTT and 5% glycerol (5-10 mg/ml packed resin). Flow-through fractions were collected and loaded on a column of DEAE-cellulose. The column was washed and eluted with the same buffer containing 0.2 M KCl and 0.32 M KCl respectively. The 7S particle fraction was adjusted to 30 % glycerol and less than 0.16 M KCl. The protein concentration was between 1-2 mg/ml as measured by the method of Bradford (13) using bovine serum albumin as a standard and more than 90% pure as judged by SDS-PAGE (14). This complex was stable at -20°C for more than one year as determined by agarose gel electrophoresis(see below).

Preparation of TFIIIA

TFIIIA was isolated by loading purified 7S RNP particles onto a Bio Gel HT column (5 mg of protein/ml packed resin) equilibrated in 10 mM phosphate buffer pH 7.5, 25% glycerol. The column was washed with 10 mM phosphate buffer pH 7.5, 25 mM Hepes pH 7.5, 0.5 mM DTT, 0.1 M KCl and the free TFIIIA was eluted with the same buffer containing 1 M KCl. The TFIIIA-containing fraction was adjusted to 50% glycerol and 0.5 M KCl and stored at -70°C. We found this method particularly suitable to study TFIIIA inactivation by alkaline buffer dilutions (see below), since it yielded highly concentrated TFIIIA.

Preparation of 5S RNA

5S RNA was purified using an extension of the procedure of Dignam et al. (15) for the purification of TFIIIA. Following elution of TFIIIA from the DEAE-cellulose with urea, the column was washed with 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA and then 5S RNA was eluted with the same buffer containing 0.5 M KCl. The eluted sample was dialyzed against 10 mM Tris-HCl pH 7.5 for 2 hours, followed by extraction with phenol-$CHCl_3$ (1:1),then with $CHCl_3$ and finally precipitated with ethanol.

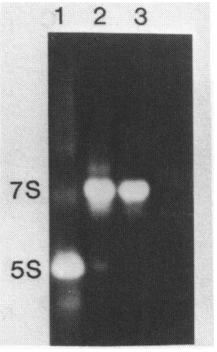

Figure 1. Effect of pH on the dissociation of 7S particle. Native 7S particles were diluted to a concentration of 50 ng/ul in Tris-HCl buffers of various pH for a period of 48 hours and the dissociation levels were determined by agarose gel electrophoresis as described in Materials and Methods. Lanes 1, 2 and 3 correspond to the samples diluted in Tris-HCl buffers adjusted to pH 8.5, 7.5 and 6.8 respectively.

Agarose gel electrophoresis

1.8% agarose gels were equilibrated and run in 50 mM Tris-Borate pH 8.3, 1 mM EDTA at 4°C for 2 1/2 hours at 10 V/cm. Gels were photographed under U.V. illumination after staining with ethidium bromide (0.4 ug/ml). No dissociation of the 7S particle occurs during electrophoresis under these conditions, even though this pH is close to the one used to inactivate TFIIIA (see below).

Inactivation of TFIIIA

Inactive TFIIIA was prepared using either the 7S particle or pure TFIIIA preparations. The inactivation can be achieved by a 1:10 v/v dilution or by dialysis against 50 mM Tris-HCl pH 8.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40 and 5 % glycerol at 4° C. The dialysis or dilutions were carried out for 48 hours at a protein concentration between 50-100 ng/ul for 7S RNP particles and at 100-200 ng/ul for purified TFIIIA preparations.

Reactivation of cross-linked TFIIIA

Reactivation reactions were carried out at room temperature in a final volume of 15 ul using, 50 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1% v/v NP-40, 5% glycerol buffer and included reduced thioredoxin. 7S denatured particles were reactivated using between 600 and 800 ng of 5S RNA. Pure TFIIIA was reactivated at a concentration of 0.8 uM and 5S RNA was added to a 1:1 molar ratio to TFIIIA. Samples were loaded onto agarose gels without the addition of tracking dye. Thioredoxin was reduced just before addition

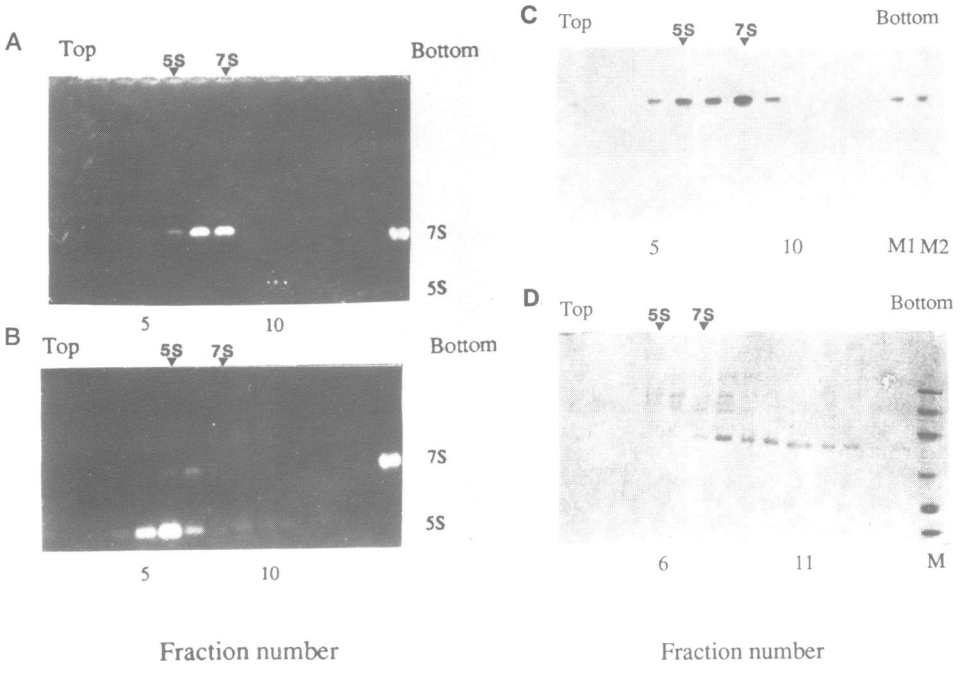

Figure 2 . Glycerol Gradient Sedimentation. Native 7S particles (panels A and C) and denatured ones at pH 8.5 (panels B and D) were sedimented through a 10-30% linear glycerol gradient at 36,000 rpm in a SW 50.1 rotor for 20 hours at 5°C. Aliquots from the collected fractions were subjected to agarose gel electrophoresis as described (A-B) or 12.5% SDS-PAGE (C-D) and stained with Coomassie Blue. Lanes M_1 and M_2 are TFIIIA and 7S particles. Lane M: low molecular weight markers (Bio-Rad) corresponding to molecular weights, M_r from top to bottom of 92,500 66,200 45,000 31,000 21,500 and 14,400. The positions of 7S particles in gradient A and 5S RNA in a parallel gradient were used as sedimentation coefficient markers.

to the reactivation reactions by incubation at room temperature with DTT for 15 minutes in the same buffer.

RESULTS

The primary method used in this work to study the interaction between TFIIIA and 5S RNA was non-denaturing agarose gel electrophoresis. This method is based on the differential electrophoretic mobilities of nucleic acid-protein complexes relative to free nucleic acid (16). At low ionic conditions and 4°C no dissociation of native 7S particles was observed during the run, which makes this a suitable method to analyze thermodynamic and kinetic parameters of TFIIIA-nucleic acid interactions (Yan Yan Xing et al.,

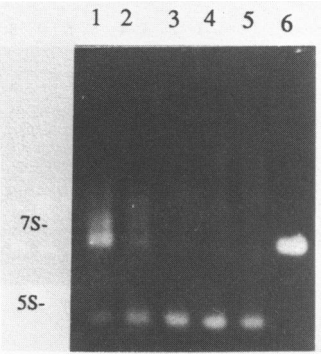

Figure 3. Effect of DTT on the denatured 7S particles. Native 7S particles denatured as described in Materials and Methods were incubated with DTT for 1 hour at room temperature. Lanes 1 through 5 represent 2, 0.1, 0.01, 0.001 and 0 mM DTT in the incubation, lane 6 native 7S particles.

in preparation). The 7S nucleoprotein complex can be dissociated as a result of the pH conditions. Figure 1 shows the effect of dilution of the native 7S particles with buffers of varying pH. At pH 6.8 and 7.5 very little dissociation of the diluted 7S particle after 48 hours at 4°C is observed. Dilution in a pH 8.5 buffer, on the other hand, results in total dissociation of the 7S RNP. As can be seen in lane 1 of figure 1 almost 90% of the nucleic acid runs as free 5S RNA with the remainder migrating as a smear in positions corresponding to higher molecular weight. We analyzed the 5S RNA and protein profile in samples dissociated at pH 8.5 using glycerol gradient centrifugation. Figure 2 presents the sedimentation profile of the 5S RNA visualized in an agarose gel. Part A corresponds to the native 7S complex and part B to the 7S particles denatured at pH 8.5 as described. Parts C and D represent parallel patterns of the protein analyzed by SDS-polyacrylamide gels of the same gradients. Controls with the native 7S particle show the 5S RNA and the TFIIIA co-sediment (Fig. 2A and C). In the case of the denatured 7S particles, however, the RNA sediments at the 5S position, and the TFIIIA sediments separately as a wide peak with an apparent S value between 6 and 12.

The broad distribution of TFIIIA in the heavier region of the gradient, instead of the normal 2.7S position of the monomer TFIIIA (17) suggested that this TFIIIA is a mixture of different populations of aggregated molecules. The fact that TFIIIA has 24 cysteine residues (18) led us to think that this aggregation might be a consequence of an oxidative process involving the sulfhydryl groups.

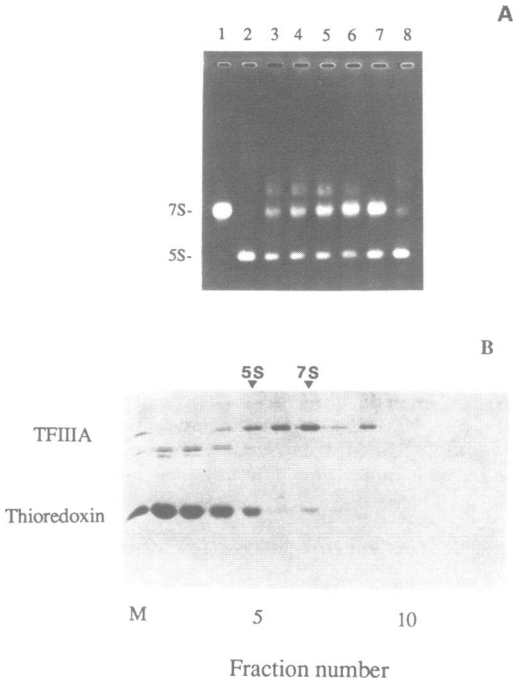

Figure 4. Reactivation of TFIIIA by thioredoxin. Denatured 7S particles were incubated with 40 uM thioredoxin (8 uM as a reduced state) for different times and then subjected to agarose gel electrophoresis as described in Materials and Methods. Panel A, lane 1: native 7S particle. Lane 2-7 represent 0. 10, 30 and 60 minutes, 2 hours and 25 hour incubation times, respectively. Lane 8 samples were incubated by 60 minutes with 40 uM DTT instead of thioredoxin.Panel B represents a sample incubated under the same conditions as panel A lane 5 which was sedimented in a glycerol gradient in the same conditions described in legend of figure 2.Aliquots of the collected fractions were subjected to 12.5% SDS-PAGE and stained with Coomasie Blue. Lane M, mixture of TFIIIA and thioredoxin standards. The positions of 7S particle and 5S RNA in parallel gradients were used as sedimentation coefficient markers.

Denaturation of the 7S particle is due to oxidation of sulfhydryl groups

To study the possibility of intermolecular disulfide bridge formation in the aggregation and subsequent inactivation of TFIIIA, as measured by its ability to bind 5S RNA, denatured 7S particles prepared by dilution with alkaline buffer (see above) were treated with DTT. Figure 3 shows the effect of DTT on the denatured 7S particle. At a 2 mM concentration, DTT was able to reactivate more than 80% of TFIIIA.

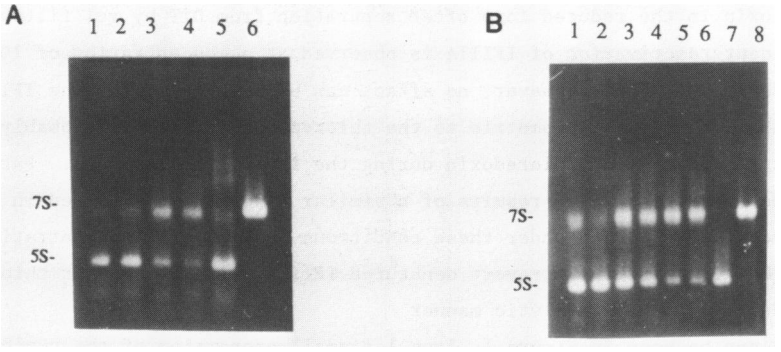

Figure 5. Reactivation of TFIIIA by catalytic amounts of thioredoxin. Panel A, Denatured 7S particles were incubated for 45 minutes with reduced thioredoxin separated from DTT by a Sephadex G-25 column equilibrated with 50 mM Tris-HCl pH 7.5, 5% glycerol and saturated with N_2. Lanes 1-4 represent 0.1, 1, 10 and 20 uM of reduced thioredoxin. Lane 5 represents 20 uM oxidized thioredoxin, lane 6 native 7S particle. Panel B, lanes 1-2 are control without thioredoxin but containing 100 and 20 uM DTT respectively. Lanes 3-6 similar experiment as lanes 1-4 of panel A, but with the addition of 20 uM DTT, lane 7 represents denatured 7S particle, lane 8 is native 7S particle.

It has been recently reported (19) that thioredoxin, a small ubiquitous protein with two redox active cysteine residues which have the ability to reduce protein disulfide bonds, can catalyze the refolding of denatured pancreatic RNase. This refolding can occur either from the reduced form or from the scrambled oxidized form of the RNase. For that reason we decided to study the effect of thioredoxin on the reactivation of TFIIIA. Figure 4 shows a time course of the incubation of denatured 7S particles with a fixed concentration of thioredoxin. Already at 10 minutes there is a detectable effect with only 8 uM of reduced thioredoxin. Part B of the same figure shows the sedimentation velocity profile of TFIIIA after incubation with thioredoxin under the conditions of lane 5 of panel A. TFIIIA now sediments as a peak at the 7S position. The minor bands that migrate in the SDS gel between TFIIIA and thioredoxin are contaminants present in the thioredoxin preparation. The results shown in figures 2, 3 and 4 indicate that after denaturation of the 7S particle TFIIIA exists as an heterogeneous population of molecules containing intermolecular disulfide bonds.

Thioredoxin reactivates TFIIIA by acting as a true catalyst

When thioredoxin is used in the oxidized form, it can not reactivate TFIIIA (Fig. 5A, lane 5). Lanes 1-4 from figure 5A shows the effect of using

thioredoxin in the reduced form after separation from DTT by gel filtration. Significant reactivation of TFIIIA is observed at a concentration of 10 uM of thioredoxin. At 1 uM, however, no effect can be seen even when the TFIIIA concentration was stoichiometric to the thioredoxin. This was probably due to fast reoxidation of thioredoxin during the isolation procedure. Part B of the same figure shows the results of a similar experiment performed in the presence of 20 uM DTT. Under these conditions, thioredoxin concentrations as low as 0.1 uM efficiently revert denatured TFIIIA, indicating that thioredoxin is acting in a catalytic manner

As can be seen in Figure 1, lane 1 a small proportion of the nucleic acid does not migrate as a free 5S RNA but as a higher molecular weight form. This observation led us to wonder if some non-specific binding of the 5S RNA was occurring which played a specific role in the denaturation of the 7S RNP particle. To address this question the experiments described above were repeated using preparations of pure TFIIIA. These preparations were also inactivated upon dilutions in buffer pH 8.5 and subsequently renatured by treatment with thioredoxin, ruling out the possibility of a specific role for 5S RNA in the oxidation and inactivation of TFIIIA (data not shown).

DISCUSSION

In the experimental results described above, we present evidence to support an oxidative process as one possible way to inactive TFIIIA. We have focused on this aspect in studying the nature of the inactive protein. When a native 7S particle is subjected to pH 8.5 conditions, it readily dissociates (Fig. 1). Nevertheless, assuming an average value for the dissociation constant (Kd) of 10^{-9} M^{-1} (20; Yan Yan Xing et al. manuscript in preparation), not more than 2% dissociation should be expected under the experimental conditions described above. This dissociation could be due to an alteration in the TFIIIA, rendering it unable to bind the 5S RNA. This conformational change then shifts the equilibrium towards complete dissociation of the 7S complex. This idea is shown in equation:

$$7S \longleftrightarrow 5S\ RNA + TFIIIA\ (a)\ active \downarrow TFIIIA\ (i)\ inactive$$

The TFIIIA in the denatured 7S particle sediments with a wide range of S values, which indicates that the protein has aggregated. The reactivation of

this cross-linked TFIIIA by DTT and thioredoxin reveals the disulfide bridge nature of this aggregate (Figs. 3 and 4). Thioredoxin is a thiol protein that has been used recently (19) in protein folding studies on pancreatic RNase. This protein is much more efficient than DTT for reactivating the denatured TFIIIA, roughly 2,000 fold (comparing lane 1 from figure 3 with lane 4 from part B, figure 5) and works as a true catalyst (Fig. 5). Significant effects are produced at a molar concentration of only 10% of the total TFIIIA concentration, provided DTT is present in the incubation. As has been previously reported (21), DTT can be used to reduce thioredoxin instead of using the in vivo system composed of NADPH and thioredoxin reductase. This reduction, which was studied by stopped flow fluorescence, is extremely fast, and this recycling of the thioredoxin which gets oxidized as a consequence of the reduction of disulfides bridges may explain the catalytic role of thioredoxin in our system. After reduction by thioredoxin the TFIIIA seems to be fully active as can be determined by the sedimentation velocity of the renatured 7S particle (Fig. 4B). This process of inter-molecular disulfide bond formation is primarily dependant on alkaline pH conditions, very likely because at this pH the anion thiolate is favored (23). The fact that we were able to reactivate the TFIIIA to nearly the same level with either DTT or thioredoxin , even when different concentrations of both are necessary, suggests that thioredoxin is reactivating the crosslinked TFIIIA by reducing protein disulfide bridges rather than acting as a protein disulfide isomerase like in the refolding of the RNase (19). Currently, however there are insufficient data to rule out the possibility of a combination of both activities in the reactivation of TFIIIA. In immature Xenopus oocytes, 5S RNA is stored associated with TFIIIA in the form of a 7S RNP complex, presumably to be used later for ribosome biogenesis. It was described (23) that ribosomal protein L_5, forms a 7S nucleoprotein complex with 5S RNA. Based on the similarities in the binding sites of Factor IIIA and ribosomal protein L_5 on the 5S RNA these authors suggested that prior to ribosome assembly, TFIIIA be displaced by protein L_5 and that this new 7S RNP complex is the one that is incorporated into the ribosome. There exists the possibility that this potential exchange of 5S RNA from TFIIIA to ribosomal protein L_5, occurs through the oxidation of TFIIIA. Studies done by others workers (10,11,15) show that TFIIIA can be thought of as being an elongated and highly asymmetrical molecule composed by 9 repetitive subdomains of 30 amino acid residues each of which has the potential capacity to bind one atom of Zn. The removal of this Zn destroys the site specific DNA binding

activity. The structure of Zn binding "fingers" was reported to be very stable and can not be formed if the cysteine pairs are oxidized forming a disulfide bridge (24). At the present time we have not identified which cysteines are involved in the formation of this disulfide bridge network of TFIIIA. Finally we want to point out that another DNA binding protein; the glucocorticoid receptor, also with Zn finger domain structures (25) is activated in rat liver cytosol by an endogenous thioredoxin system (26).

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ABBREVIATIONS

TFIIIA, transcription factor III A; DTT, dithiothreitol.

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