Soluble and Insoluble Rat Liver Chromatin
is Different in Structure and Protein Composition

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Rat liver chromatin has been fractionated by different solubility in solvents of 155 mM ionic strength in soluble S and insoluble I-chromatin. Histone H1 content is lower in S as compared to I-chromatin. The HMG1/2 nonhistone proteins are observed in S-chromatin and in the nuclear pelleted residue from the chromatin isolation procedure, but no amount can be detected in I-chromatin. Thermal denaturation profiles and CD-spectra are different for S and I-chromatin indicating distinct interactions between DNA and proteins in the chromatin molecules. Both effects, differing protein content and distinct DNA-protein interactions, can be correlated with solubility and insolubility being the result of charge-charge interactions between chromatin molecules and ionic components of the solvent.

Introduction

It is a well established fact that eukaryotic chromatin is organized in nucleosomes as a structural subunit [1, 2]. At low ionic strength the nucleosomal chain is extended as "beads on a string" [3]. With increasing salt concentration in the solvent the chain is continuously folded to a compact quaternary structure [4—10], which is described as a solenoid [11] or as an arrangement of superbeads [12].

In solvents of about physiological or slightly higher ionic strength chromatin becomes insoluble [13—16]. However, isolation procedures have been developed, which yielded soluble high molecular chromatin in solutions at nearly physiological ionic strength [17—20]. Solubility and insolubility of chromatin is apparently a property being very sensitive to the composition of the solvent and to the DNA-protein combination of the chromatin molecules and the resulting interaction between these components.

If chicken erythrocyte chromatin is isolated in medium salt concentration, it can be fractionated by increasing the ionic strength into a part remaining soluble (S-chromatin) and another part (I-chromatin), which becomes insoluble and precipitates [21—23]. Several differing properties have been reported for these two kinds of chromatin. There are also attempts to correlate solubility and other physico-chemical properties to gene activity [24—25].

In this paper fractionating of rat liver chromatin into S and I-chromatin is described, which differ with respect to absorbance and CD-spectra, thermal denaturation, amount of H1 histones and HMG1/2 nonhistone proteins and properties of extracted DNA.

Materials and Methods

Chromatin isolation and fractionation into S and I-chromatin

Cell nuclei were isolated from rat liver as described by Pogo et al. [26], all solutions containing 0.5 mM PMSF. Soluble chromatin was isolated by a modification of an autodigestion procedure which was first reported by Hewish and Burgoyne [27] using endonuclease activity stimulated by calcium and magnesium. The nuclei of about 35 g liver were washed twice and then incubated for 10 min at 37 °C in three portions each containing 0.5 ml of the digestion buffer (40 mM NaCl, 15 mM KCl, 3 mM MgCl2, 1 mM CaCl2, 0.14 mM spermidine, 0.2 mM PMSF, 5 mM Tris-HCl, pH 7.5). The incubation was stopped by cooling on ice and adding 0.02 ml 0.1 M EDTA and 0.02 ml 0.1 M EGTA. The nuclei suspension was then dialyzed overnight at 4 °C against a dialyzing buffer (45 mM NaCl, 15 mM KCl, 0.4 mM MgCl2, 1 mM EGTA, 0.2 mM PMSF, 5 mM Tris-HCl, pH 7.5) with an ionic strength of 68 mM. After cen-

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trifugation at 4000 × g (5 min, 4 °C) the supernatant contained the soluble chromatin. The sedimented residue was suspended in 2 ml of the dialyzing buffer and dialyzed again for 3 h at 4 °C against the same buffer. Centrifugation led to some additional soluble chromatin which was added to the first fraction giving about 4 ml chromatin solution with an absorbance near 100 at 260 nm. The sedimented pellet P was extracted as described later to compare the DNA, the histones and the HMG proteins with those of the two chromatin fractions.

In order to fractionate into soluble S and insoluble I-chromatin the solution was dialyzed overnight or for 4 h at 4 °C against the fractionating buffer (112.5 mM NaCl, 37.5 mM KCl, 0.25 mM MgCl₂, 0.63 mM EGTA, 0.13 mM PMSF, 3.13 mM Tris-HCl, pH 7.5) with a nearly physiological ionic strength of 155 mM. As an alternative procedure fractionating was done by adding appropriate volumes of a solution with higher salt concentration. Centrifugation at 4000 × g (5 min, 4 °C) separated the soluble S-chromatin in the supernatant from the precipitated insoluble I-chromatin. If necessary the latter was redissolved in a buffer (10 mM NaCl, 0.4 mM MgCl₂, 1 mM EGTA, 0.2 mM PMSF, 5 mM Tris-HCl, pH 7.5) with the low ionic strength of 18 mM.

Sedimentation coefficients

Sedimentation coefficients of chromatin or isolated DNA were determined using standard techniques at 20 °C in an analytical ultracentrifuge (Mark II from MSE Ltd., Crawley, UK). Relative molecular masses \( M_r \) of the chromatin molecules were estimated from 

\[ s \] 

-values according to data of Butler, Thomas and Bates [28–30], \( M_r \) values of isolated DNA were calculated by the formula of Triebel [31].

Circular dichroism

CD-spectra of chromatin solutions were taken with a spectropolarimeter (J-500A from JASCO, Tokyo, Japan) at 25 °C in 1.0 cm path-length cuvettes. Measurements were repeated four times. Sample concentration was determined by absorbance measurement at 260 nm using a molar absorption coefficient of \( \varepsilon_{260} = 6600 \) l/cm M for a nucleotide. Ellipticity was monitored between 255 and 320 nm, a range which is sensitive only to the DNA part of chromatin.

Isolation and characterization of histones and HMG nonhistone proteins

Histones were extracted from the nuclear pellet P and the chromatin fractions S and I near 0 °C with 0.2 M H₂SO₄, containing 0.05 M NaHSO₃. They were characterized by electrophoresis on urea/acetic acid polyacrylamide gels according to Panyim and Chalkley [32]. The gels were stained with Coomassie Blue and the obtained histone pattern scanned at 550 nm with a gel-scan spectrophotometer (Gilford System 2600 from Ima Gilford, Giessen, FRG).

The acid-soluble nonhistone proteins were extracted from the nuclear pellet P and the chromatin fractions S and I by a modification of a method described by Rabbani et al. [33]. The sample was extracted three times with 5% HClO₄, the extracts were combined with 0.03 volume of conc. HCl. In order to precipitate the proteins 8 volumes of ice-cold acetone were added. After storage overnight at −20 °C centrifugation at 2000 × g (15 min) led to precipitated proteins being histone H1 and HMG nonhistone proteins. Further purification was done by dissolving in 0.1 M HCl and precipitating again with 8 volumes ice-cold acetone. Attempts to separate most of the histone H1 by fractionated precipitation with 2 to 4 volumes acetone were not successful. The proteins were subsequently analysed by gel-electrophoresis and scanned as described above.

To make sure that the extracted proteins from S-chromatin are really chromatin-bound and not only accompanying molecules, as an alternative procedure S-chromatin was pelleted by centrifugation (Beckman SW65 rotor in a Kontron Centrikon T2070 ultracentrifuge for 5 h at 55000 rpm and 4 °C) and subsequently extracted. The protein patterns were identical for the extracts obtained by the two methods.

Isolation of DNA

DNA was extracted from the nuclear pellet P and the chromatin fractions S and I by standard procedures [34]. The ethanol-precipitated DNA was redissolved in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate). The ratio of absorbances \( A_{260}/A_{280} \) was 0.51–0.54.

Thermal denaturation

DNA samples were thermally denatured in 0.1 × SSC at a concentration corresponding to an ab-
sorbance of about 0.4 at 260 nm with a temperature increasing rate of 0.5 °C/min. Absorbance at 260 nm and cuvette temperature were simultaneously monitored for three samples and the buffer as reference by a spectrophotometer with temperature-controlled cuvettes and a thermo-programmer (Gilford System 2600). The data-processor of the spectrophotometer was used for derivative calculation and averaging. The denaturation profiles were presented as the derivative of sample hyperchromicity with respect to temperature \( dA_{260}/dt \). The melting point \( t_m \) was defined as the temperature of the profile maximum.

Chromatin samples were thermally denatured in a low ionic strength buffer (0.2 mM EDTA, 1 mM NaH\(_2\)PO\(_4\), pH 7.0) at conditions described above.

**Results**

**Chromatin properties**

The described autodigestion procedure in the 68 mM buffer with an incubation time of 10 min was the result of experiments (not shown) obtaining the optimum with respect to yield and molecular size of soluble chromatin. Shorter incubation reduced the yield, and at longer incubation time the relative amount of mono and dinucleosomes increased, while that of oligonucleosomes decreased. Considering the fact that mononucleosomes in general are soluble in higher ionic strength solvents, the ratio of S-chromatin to I-chromatin was found to be nearly independent on the incubation time.

The amount of soluble rat liver chromatin obtained by the described auto-digestion procedure in the 68 mM buffer was 50 to 70% of the whole chromatin within the nuclei. The sedimented residue contained insoluble and matrix-bound chromatin, components of the nuclear membrane and nuclear debris. The fractionation procedure in the 155 mM buffer yielded 20 to 35% S-chromatin and 65 to 80% I-chromatin. Comparison of absorbance spectra in the wavelength range 220–320 nm indicated some more proteins in S-chromatin (\( A_{260}/A_{280} = 1.55–1.65, A_{260}/A_{230} = 0.68–0.75 \)) as compared to I-chromatin (\( A_{260}/A_{280} = 1.75–1.80, A_{260}/A_{230} = 1.49–1.55 \)).

Thermal denaturation of chromatin solutions in the EDTA/phosphate buffer (Fig. 1) produced lower hyperchromicity of S-chromatin as compared to I-chromatin. The melting point \( t_m \) was 79.24 ± 0.48 °C for S-chromatin and 80.11 ± 0.20 °C for I-chromatin (mean values with S. D. from 3 experiments). The denaturation profile of I-chromatin was more asymmetric with an indication of a slight shoulder in the curve at 75–76 °C.

From analysis of the sedimentation data a very broad s-value distribution was obtained for both S and I-chromatin. In the buffer of 68 mM ionic strength I-chromatin had sedimentation coefficients from 35 to 65 and S-chromatin from 30 to 40 indicating lower relative molecular masses for S-chromatin molecules. This was confirmed by some electrophoretic tests on 2% agarose gels employed to chromatin samples being denatured by Sarkosyl [35]. The ethidium bromide stained gels showed higher molecular DNA in I as compared to S-chromatin, in the latter even some mononucleosomal DNA (K. Hartmann and H. Merkel, unpublished data).

**Circular dichroism**

Fig. 2 shows different CD-spectra for S and I-chromatin in ionic strength solvents of 68 mM as well as in 0.2 mM EDTA solution (ionic strength 0.6 mM). In general the positive ellipticity between 260 and 295 nm increased with decreasing ionic strength. In both media the maximum, however, was at 275 nm for S-chromatin and at 283 nm for I-chromatin. This significant difference was demonstrated more clearly by the ratio of the ellipticities at 283 and 275 nm. This value \( 0.283/0.275 = 0.84 ± 0.07 \) in 68 mM solvent and \( 0.82 ± 0.06 \) in 0.6 mM solvent for S-chromatin, but 1.31 ± 0.09 at the higher and 1.22 ± 0.09 at the lower ionic strength for I-chromatin (the given deviation is S. D. from 4 measurements).
Fig. 2. CD-spectra of S (-----) and I-chromatin (--------) in 68 mM ionic strength buffer (A) and in 0.2 mM EDTA (B). The buffer in (A) contains 45 mM NaCl, 15 mM KCl, 0.4 mM MgCl2, 1 mM EGTA, 0.2 mM PMSF and 5 mM Tris-HCl with pH 7.5.

Histones and HMG proteins

In Fig. 3 gel-scans of the histone patterns of I and S-chromatin are compared. The relatively lower peak height of histone H1 in S-chromatin indicated a lower content of H1 as compared to I-chromatin. The ratio of the peak heights of H1 to H3 was 0.97 ± 0.05 for S-chromatin and 1.26 ± 0.13 for I-chromatin (deviation was S. D. from 4 measurements). Comparing this peak height ratio from the histone pattern of the sedimented pellet P to that of whole chromatin (not shown), a slightly lower content of H1 in chromatin was apparently caused by leak of H1 in the S-chromatin fraction.

Using the extraction procedure for isolation of HMG proteins, in addition a large amount of histone H1 was isolated as demonstrated in Fig. 4, in which gel-scans of the protein pattern of HMG extracts from I and S-chromatin are compared with that of the nuclear pellet P. As marker the histone pattern of chromatin (H) and an HMG extract from rat thymus (T) according to Rabbani [33] was added. Left from the histone H1, which was the main peak in all HMG extracts, a broad peak indicated in the pellet P as well as in S-chromatin a significant amount of the nonhistone proteins HMG1/2, which could not be detected in I-chromatin. Due to low...
resolution of the gels HMG 1 and 2 were only slightly in the rat liver pattern, but the position of the bands was in agreement with data reported in the literature [33].

Chromatin DNA

Thermal denaturation as derivative profiles of isolated DNA from S and I-chromatin and the nuclear pellet P were plotted as shown in Fig. 5. Hyperchromicity of I-chromatin DNA was higher as compared to that of S-chromatin and pellet P. Melting point $t_m$ was lower for S-chromatin DNA in comparison to those of I-chromatin and pellet P. Mean $t_m$ values (with S. D. from 9 experiments, some measured twice) of the DNA from S-chromatin were 66.92 ± 0.26 °C, from I-chromatin 67.57 ± 0.20 °C, from pellet P 67.73 ± 0.24 °C. The difference between the latter two values was in the range of the S. D., so it was not significant.

Analysis of sedimentation experiments with the isolated DNA showed very broad s-value distributions. When calculating the relative molecular masses lower values were obtained for S-chromatin DNA in the range of $(0.5-3) \times 10^5$, while I-chromatin and pellet DNA gave higher values, $(3-7) \times 10^5$ and $(2-5) \times 10^5$, respectively.

Discussion

Fractionation of chromatin due to different solubility in solvents at about physiological ionic strength has been described for chicken erythrocyte chromatin [21—23, 25]. In this paper results of fractionating chromatin from rat liver cell nuclei at similar conditions are reported.

The striking difference between S and I-chromatin is the solubility or non-solubility in solvents of defined higher ionic strength. Chromatin molecules are negatively charged due to incomplete neutralization of the negative charges of the DNA phosphates by basic, positively charged proteins [36, 37]. In solvents of lower ionic strength chromatin structures are extended due to the repelling forces of the charges on the surface of the nucleosomes. With increasing ionic strength unspecific charge neutralization by the monovalent cations reduces the repelling forces and internucleosomal interactions induce chromatin condensation to higher order structures [11, 12]. Divalent cations have higher efficiency [5, 6, 16, 38] due to a specific binding which has been demonstrated for Ca$^{2+}$ in the following paper [39]. In solvents of about 150 mM monovalent salt concentrations charge neutralization is nearly perfect, thus uncharged molecules aggregate and precipitate, and chromatin becomes insoluble.

On the one hand this effect is very sensitive to the exact composition and concentration of the solvent, and on the other hand to the charge density at the surface of chromatin molecules. The latter is a result of the composition of DNA with histones and other basic proteins forming chromatin, and the nature of the interactions between these components. Both have been found to be different for S and I-chromatin. The amounts of histone H1 as well as that of the HMG proteins are not identical. Differing DNA-protein interactions in the chromatin fractions can be elucidated from the differences of the thermal denaturation behaviour and the CD spectra.

Slightly reduced content of H1 histone in S-chromatin, found for rat liver chromatin, has also been reported for chicken erythrocyte chromatin [23, 25]. Therefore, S-chromatin molecules have a higher charge density due to a lower degree of neutralization. The content of HMG proteins in S-chromatin is very low and does not compensate the leak of H1. Additionally, the binding of HMG proteins to the nucleosomes may be not only a simple charge-charge interaction [40], thus a higher content of these proteins does not necessarily decrease charge density. One can postulate that a low amount of HMG proteins in chromatin induces changes of the interactions between DNA and proteins, histones as well as HMGs. These interactions are different in the two
chromatin fractions as HMGs are components of S-chromatin, but not of I-chromatin. The result is a different charge density for the two kinds of chromatin, and therefore a different sensitivity to being precipitated by a certain salt concentration.

Two additional observations indicate that there are different DNA-protein interactions in S and I-chromatin: thermal denaturation of chromatin and CD-spectra. It has been reported that free DNA has lower $t_m$-values as that being complexed with proteins [41—43]. Although there is a shoulder at lower temperature in the thermal denaturation profile of I-chromatin, its $t_m$-value is higher as compared to that of S-chromatin. From this behaviour collectively a stronger binding between DNA and the proteins (histones) can be elucidated. This stronger interaction is equivalent to a higher degree of charge neutralization in I-chromatin.

The CD-spectra of the chromatin solutions between 260 and 300 nm show similar ellipticities as those, which have been reported by several authors [23, 42, 44—47] for oligonucleosomes. Free DNA has very high positive ellipticity of 7700—8600 deg·cm²/dmol, while DNA, which is complexed with proteins in a maximum way (nucleosome core particles), shows low values of only 400—1300 deg·cm²/dmol. The slight increase of chromatin ellipticity with decreasing ionic strength of the solvent is also in agreement with reported data [23, 47]. The striking difference in the CD-spectra of S and I-chromatin is the ratio $0.283/0.275$, which has been observed for chicken erythrocyte S and I-chromatin too [23]. The reason of this phenomenon is unclear, but one can postulate a different kind of DNA-protein interaction from the different shape of spectra of S and I-chromatin in agreement with the chromatin denaturation studies.

The DNAs, isolated from the two chromatin fractions, are different. S-chromatin DNA has lower relative molecular masses as compared to I-chromatin DNA. The DNA, extracted from the nuclear pellet, is similar to that of I-chromatin. $M_r$ values of chromatin DNA in general are lower than those being expected from the molecular masses of the whole chromatin molecules (calculated from $s$-values). This discrepancy can be explained by double-strand breaks within the DNA of the chromatin molecules cut by nuclease at the isolation procedure [48]. Sensitivity to endogenous nucleases is apparently higher for S-chromatin.

Thermal denaturation of DNA indicates a significantly lower $t_m$-value for S-chromatin as compared to I-chromatin and pellet P. This lower value could not be explained by a lower GC-content in S-chromatin, as sedimentation equilibrium experiments in CsCl-gradients gave no lower buoyant density of the S-chromatin DNA. The reason for the lower $t_m$-value may be the lower relative molecular mass of DNA from S-chromatin. A dependence of the $t_m$-values to $M_r$ of DNA molecules with identical base mixture in a certain range of molecular masses has been reported [43, 49, 50]. An alternative explanation for the differing $t_m$-values may be distinct impurities of residual proteins in the DNA, which could not be eliminated by standard procedures.

Preliminary DNA/RNA hybridization results indicate higher transcriptional activity in S-chromatin and pellet P as compared to I-chromatin (not published). This is in agreement with the higher content of HMG-proteins, a certain deficiency of H1 histones and an increased nuclease sensitivity, which have been correlated to higher activity [8, 25, 51, 52].

One may argue that different solubility of chromatin in solvents of physiological ionic strength and the other observed differences have no biological relevance and are produced as artefacts due to the isolation and fractionation procedure. Especially, the leak of histone H1 in S-chromatin could be suspected as a result of the isolation procedure [53]. However, the sum of the results as a whole indicate a biological relevance.

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