The solubility of calf thymus chromatin in sodium chloride

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ABSTRACT

The solubility of calf thymus chromatin and chromatin depleted of F1-histone has been examined under various conditions in sodium chloride. F1-depleted DNH was more soluble than native DNH at low concentrations but this difference became small at high concentrations (1mg/ml). Both exhibited minimum solubility in 0.15M -NaCl. The effect of pH and of maleylation of the amino acid side chains on the solubility implied that electrostatic interactions dominated the precipitation reaction. Urea had no effect on the solubility of either complex. N.m.r. studies showed that the chromatin behaved as a rigid complex at all salt concentrations less than 0.6 molar.

INTRODUCTION

It is well known that chromatin has a pronounced solubility minimum in 0.2M -NaCl. This property has formed the basis of many procedures for isolating chromatin from nuclear and whole cell lysates. It has also led to the suggestion ^{1,2} that precipitation of chromatin in such solutions, may be similar to the in vivo process giving rise to the condensed chromatin observed in some interphase cells and also to the condensation which occurs at metaphase. Several studies ^{2,3} have suggested an important role for the lysine-rich histone F1 in this process and recently a more specific mechanism involving interactions between different F1 molecules has been proposed ². In view of the important implications which these findings have for the structure of the chromosomal material in both interphase and metaphase cells we have investigated the solubility properties of chromatin under a variety of different solution conditions paying particular attention to the role of histone F1. The results are presented below.

EXPERIMENTAL

a) Preparation of material

Calf thymus nucleohistone (DNH) was prepared by the method of Zubay and Doty⁴. Histone F1-depleted material was prepared as described elsewhere⁵. Histone F1 was prepared by dissociating the protein from the DNH in 0.7M -NaCl and separating the components by gel filtration on Sepharose 4B equilibrated with 0.7M -NaCl. Methods used for the physical and chemical characterisation of all preparations have been described^{5,6}. All solutions contained 0.7mM sodium phosphate, pH 6.8, unless otherwise stated.

b) Construction of solubility curves:

Soluble nucleohistone is defined as that material which remains in solution after centrifugation at 30,000g for 20 minutes under standard conditions. Appropriate volumes of standard NaCl were added to DNH (1ml) at room temperature to give a final volume of 4 mls. After centrifugation the supernatant was removed and the percentage of DNH remaining in solution was determined from the absorbance at 260nm (A_{260}).

c) Variation of solubility with pH:

DNH solutions were titrated at room temperature by the direct addition of 2M-NaOH from a micrometer syringe. The solution was rapidly stirred and the pH was monitored with a glass electrode and a direct-reading E.I.L. pH meter calibrated with 0.05M potassium hydrogen phthalate (pH 4.01) and 0.05M sodium borate decahydrate, (pH 9.18).

d) Circular Dichroism:

Spectra were recorded to a Roussel - Jouan Dicrograph, Model CD 185, at room temperature using the procedure and precautions described previously⁶.

e) Modification with Maleic Anhydride:

The method was essentially that of Butler et al⁷. Maleic anhydride (0.05M) was added dropwise to DNH (protein conc. = 500 µg/ml) and the solution maintained at pH 8.0 by the addition of 5 N NaOH. The course of the reaction and the extent of modification was estimated spectrophotometrically from the

increase in extinction at 280nm due to the maleyl amino group. When the reaction was complete the mixture was dialysed against 0.7 mM sodium phosphate pH 6.8 to remove excess reagent and products.

f) Modification with tetranitromethane (TNM):

The nitration of the single tyrosine residue on histone F1 was performed as described by Bustin⁸. A ten-fold molar excess of TNM over tyrosine was used and the reaction monitored from the absorbance at 428nm of the nitroformate anion. The final extent of reaction corresponded to 80% modification of the tyrosine side chain. Reconstitution of whole DNH was effected by adding the correct stoichiometric amount of F1 - depleted DNH to the modified histone F1 and dialysing extensively at room temperature into 0.7mM phosphate buffer (pH 6.8).

g) Nuclear magnetic resonance:

DNH solutions were concentrated by centrifugation at 200,000g for 4hr. to a final concentration of 10mg/ml protein. The gels were dialysed against NaCl solutions made up with D₂O. High resolution protein spectra were recorded on a Bruker 270 MHz spectrometer in 5mm tubes at 20°C.

h) Polyacrylamide Gel Electrophoresis:

The method was similar to that described by Panjim and Chalkley⁹ using a final concentration of 2.5M urea in the running gel.

RESULTS

Solubility in NaCl:

The solubility curves of DNH (900 µg/ml) and F1-depleted DNH (800 µg/ml) are shown in Fig. 1. Both curves were sigmoidal and both showed a minimum at 0.15M -NaCl, the solubility increasing again above 0.2M -NaCl as histones became progressively more dissociated from the DNA. At the minimum, in 0.15M -NaCl, 95% of the native DNH was insoluble compared with 73% of the F1-depleted sample. In both cases the precipitate redissolved on dialysis into low phosphate buffer.

The solubility in 0.15M -NaCl was very dependent on concentration (Fig.2). At low concentrations ($A_{260} = 0.075$)

Fig. 1 Solubility of nucleohistone in salt □-□-□ whole nucleohistone, pH6.8; ■-■-■ whole nucleohistone, pH6.8 6M urea; ●-●-● whole nucleohistone, pH8.0; ○-○-○ whole nucleohistone pH5.0; ●-●-● fl-depleted nucleohistone.

there was a marked difference between the solubilities of DNH and Fl-depleted DNH. At higher concentrations ($A_{260} = 10.0$), however, the curves tended to converge. Thus the absence of Fl renders the DNH more soluble at very low concentrations but has little effect at concentrations greater than 1mg/ml.

The effect of urea:

Identical solubility curves were obtained for both DNH and Fl-depleted DNH when the above experiments were repeated in the presence of 6M urea (Fig.1). Thus the precipitation reaction is not apparently dominated energetically by hydrophobic interactions or reactions involving hydrogen bonds.

The effect of pH:

By contrast, increasing the pH in the range 5.0 to 8.0 caused a progressive displacement of the solubility curves to

Fig 2. Dependence of the solubility of nucleohistone in 0.15M NaCl upon concentration (●-● DNH-fl ; ■-■ DNH)

higher salt concentrations without changing their shape (fig.1). Altering the net charge on the DNH therefore has a marked effect on the solubility which suggests that charge-charge interactions are important in the precipitation reaction. This effect was investigated in another way by determining the solubility of native DNH and Fl-depleted DNH in 0.15M -NaCl as a function of pH (Fig.3). Both complexes showed a sharp increase in solubility at pH values above 10.8. Visually, the precipitate completely dissolved but centrifugation resulted in a 25% loss of material from the supernatant at pH 11.0. The absence of Fl appeared to make little difference to the solubility properties of the complex (Fig. 3).

Maleic anhydride modification:

In accordance with previous observations¹⁰, maleic anhydride caused modifications to all the histone fractions

Fig. 3. Dependence of nucleohistone precipitation upon pH in 0.15M NaCl. (□-□-□ whole nucleohistone; ■-■-■ whole nucleohistone modified with TNM; ●-●-● fl-depleted nucleohistone)

in both DNH and Fl-depleted DNH as judged from the absence of any bands corresponding to the five unmodified histone fractions when the protein was electrophoresed on polyacrylamide - urea gels. Thus the lysine amino acid side chains in all five histone fractions had been modified to some extent. The modified DNH and the Fl-depleted, modified DNH were both completely soluble in 0.15M NaCl. In the modification of nucleohistone by this method the major effect is to replace the positively charged amino group on a lysine with a carboxylic acid group, which will be negatively charged at neutral pH. Thus, once again altering the net charge on the complex brings about a change in solubility properties.

Modification with TNM:

The circular dichroic spectrum of the reconstituted DNH in the 220nm region was identical to native DNH, indicating that no major change in secondary structure of the Fl

Fig 4. CD Spectra of nucleohistone treated with tetranitromethane

- a) untreated nucleohistone. b) modified nucleohistone.
c) fl-depleted nucleohistone.

histone had taken place during the dissociation-modification-reassociation process (Fig.4). However, there was a reduction in the magnitude of the positive dichroic band at 277nm, usually attributed to the DNA component of the complex. In this case, the change in dichroism may have arisen from contributions from the chemically modified tyrosine on F1 since no differences in dichroism were detected between a native sample and a reconstituted, unmodified complex.

The solubility curve of TNM - modified DNH ($A_{260}=1.04$) at pH 6.8 was identical to that of native DNH. The dependence of the solubility on pH in 0.15M-NaCl was also similar to that of native DNH (fig.3). Thus the introduction of a nitro group into the ortho position relative to the hydroxyl in the single tyrosine residue of histone F1 does not appear to alter the solubility properties of DNH.

N.m.r. studies:

The 270 MHz spectra of DNH gel in the presence of

varying amounts of NaCl are shown in Fig. 5. The absence of a signal in low salt (curve a) indicated that the molecular structure was very rigid so that the spectrum is, as expected, unobservably broad². Increasing the salt concentration to 0.01 or to 0.1Molar caused the gel to aggregate and to become opaque. Again, the absence of a signal (curve b, Fig 5) suggested that none of the histone residues were mobile in this condensed, precipitated state. This is in contrast to the observations of Bradbury *et al*² who observed the appearance of peaks which they identified as corresponding to the central portion of F1 at this ionic strength. In 0.6M-NaCl several peaks were visible in the spectrum (curve c). The overall shape and positions of the peaks were very similar to those observed by Bradbury *et al*² for F1 histone and it may be concluded that the n.m.r. spectrum at this salt concentration is due almost entirely to dissociated F1.

The spectrum in 0.1M-NaCl at pH 11.0 was similar to native DNH, i.e. featureless. Thus there was no increase in mobility of histone side chains associated with the increased solubility of the complex at high pH.

DISCUSSION

The ready precipitation of nucleohistone from solution by sodium chloride at moderate ionic strengths suggests, *a priori*, that the most important contribution to the precipitation mechanism will be one involving binding of ions to the DNH to give an electrostatically neutral complex¹. That this is fundamentally the case is amply supported by the results presented here. The increase in solubility with increasing pH above 5.0 is readily explained in terms of the net charge on the DNH. Electrometric titration studies of DNH¹¹ have shown that the isoionic and isoelectric pH is close to 5.1 and that the complex becomes insoluble below this pH in 10⁻³ M-NaCl. Clearly the DNH will be least soluble at this point and will become more so as the net negative charge increases due to ionisation of the side-chain carboxyl groups and neutralisation of the histidine residues, as the pH increases. At higher pH (10-11) the free lysine and tyrosine side chains titrate, both reactions increasing the overall net negative charge and the DNH becomes soluble even in 0.15M-NaCl. The overall net negative charge on the complex is also increased by the maleylation reaction and again one observes complete solubility of the chemically

Fig 5 High-field parts of the 270MHz spectra of nucleohistone in salt :

a) D_2O . b) $0.1M$ NaCl. c) $0.6M$ NaCl. d) $2M$ NaCl.

modified DNH in $0.15M$ NaCl at pH 7.0. These conclusions are supported by the absence of any detectable effect of $6M$ urea on the shape or the position of the solubility curves which implies that hydrophobic and hydrogen-bonding interactions are not important and play a negligible role in the precipitation reaction. Furthermore the n.m.r. studies reported here do not suggest any special function for F1 in the condensation. These observations are in marked contrast to those of Bradbury *et al*² who state that active condensation cannot take place under conditions unfavourable to hydrogen bonds and hydrophobic

interactions. They also concluded that F1 was necessary for the condensation of calf thymus DNH. Again, this is at variance with the results presented here which have demonstrated that, except at very low concentrations, the absence of F1 from the complex makes no difference to its solubility properties in salt. Furthermore, chemical modification by nitration of the one tyrosine side chain in F1 has no measurable effect on the precipitation reaction. This tyrosine residue is in the middle of a region on the F1 histone which, it has been suggested¹², may be involved in interactions with other F1 molecules which are important for the condensation reaction. Clearly, if such interactions exist, the introduction of a bulky nitro group into the aromatic ring, with the consequent decrease in pK of the hydroxyl group, does not affect them.

The studies of Jensen and Chalkley¹ and Smart and Bonner¹³ in general support our findings that the mechanism of precipitation is primarily an electrostatic phenomenon involving the neutralisation of net negative charge on the nucleoprotein complex with no one histone fraction playing a dominant role. However, the former study used a different method for the preparation of chromatin to that used here. This method produced two distinct species differing in sedimentation coefficient. The slower sedimenting species had properties closely corresponding to those of the DNH described here. On the other hand the faster sedimenting species, which may correspond to the gel fraction normally discarded in the Zubay-Doty preparation, showed aggregation properties which appeared to depend on F1 and which are irreversibly altered in urea. In the light of these studies our results and those of Bradbury *et al.*² may be reconciled if it is assumed that the latter have been studying the gel fraction of DNH. On this basis the gel fraction would have solubility properties which are significantly dependent on F1 whereas the soluble fraction, studied by us, apparently has solubility properties independent of F1. It is not clear to us whether the separation into soluble and gel fractions is artificial, arising from the Zubay-Doty preparation procedure (see the discussion by Fredericq) or whether the separation reflects a real difference between the two fractions present in vivo.

Whatever the final answer to this question the results presented here clearly show that the precipitation of the soluble chro-

matin fraction is independent of F1 and proceeds by a non-specific mechanism involving charge neutralisation of carboxyl groups on the surface of the nucleohistone. This being so, it is unlikely that the precipitation reaction can be used as a useful model for condensation of chromatin which occurs at metaphase since the non-specific nature of the charge neutralisation process could hardly give rise to the highly ordered structure present in metaphase chromosomes revealed, for example, by banding techniques¹⁵. However, charge neutralisation must certainly take place in order to allow the close packing of the chromatin threads in the condensed state but whether it is mediated by inorganic ions or protein remains to be seen. A comparison of the titration curves of nucleohistone¹¹ and metaphase chromosomes¹⁶ suggests non-histone proteins and magnesium ions may play an important role in this process.

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