On the Interaction of Histones with Polyanions

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Abstract. Histones precipitate from a solution of 0.14 mol/l NaCl with increasing concentrations of the polyanions polypentosesulphate; dextran sulphate; inorganic polyphosphate; heparin; or copolymer ethylene-maleic acid forming complexes from which histones cannot be extracted by 0.25 mol/l HCl. Affinities of the histone classes for polypentosesulphate appeared in the order from greatest to least: H4 ~ H3 > H2A > H2B > H1. At increased concentrations of most polyanions studied, the complexes of histones with polyanions remained partially soluble. Complexes of histones with all polyanions used were completely soluble in 2% SDS electrophoresis buffer, in 0.14 mol/l NaCl buffered at pH 12, and in 2 mol/l NaCl buffered at pH 7.2. Solubilisation of the complex polypentosesulphate-histone in 2 mol/l NaCl proved to be due to its dissociation.

Key words: Polyanions — Histones — Interaction

Introduction

Polyanions, when added to nuclei, cause pronounced morphological changes which can be correlated with increased DNA and RNA syntheses (Skalka et al. 1968; Berlowitz et al. 1972; Miller et al. 1972; Arnold et al. 1972; Cook and Aikawa 1973). Small polyanion concentrations lead, in the reaction with chromatin, to an increased accessibility of DNA to nucleases (Skalka and Čejková 1973), but only higher polyanion concentrations bring about changes in some of the physicochemical properties of chromatin (Skalka et al. 1976). These changes are obviously caused by the reaction of polyanions with histones. Histones are known to restrict the accessibility of enzymes to DNA in the chromatin complex. It may be suggested that natural polyanions take part in a nonspecific derepression of the genome by means of binding to nuclear chromatin histones. However, it is still not known whether the reaction of polyanions with chromatin is accompanied by the release of histones from the complex with DNA or whether, at lower concentrations of polyanions, a complex of DNA-histone-polyanion is formed (Courvalin et al. 1982). In the latter case it remains unclear whether and how the histones bound to

polyanions could be released to restore their complete binding to DNA. It is known that free histones form complexes with polyanions which resist dissociation and are insoluble at lower ionic strength (Kent et al. 1953; Matyášová et al. 1974; Kitzis et al. 1976; Hildebrand et al. 1977; Miller et al. 1971).

In this paper the interaction of free histones with different polyanions and the influence of ionic strength and pH on the stability of polyanion-histone complexes were investigated as an outset to further studies on the interaction of nuclear chromatin histones with polyanions.

Experimental

Polyanions used. Polypentosesulphate (Benechemie München; gift of Dr. T. Halse to M. S.; molecular weight 2,000); heparin pro inj. (Spofa, Czechoslovakia; molecular weight not determined); dextran sulphate (Pharmacia, Sweden; prepared from dextran of molecular weight 500,000); inorganic polyphosphate Na-salt (Albright and Wilson, England; molecular weight 4,300); and copolymer ethylene-maleic acid Na-salt (Monsanto, USA; molecular weight not indicated).

Preparation of ^{3}H-polypentosesulphate. Polypentosesulphate was labelled with tritium and purified as described by Matyášová et al. (1971). Estimation of polypentosesulphate amounts was done by means of the metachromatic reaction with Azure I as described by Skalka (1968), and radioactivity was measured as described below.

Isolation of histones. Histones were isolated from calf thymus by extraction with 0.25 mol/l HCl, precipitated in 2 mol/l trichloroacetic acid (TCA), washed in acidified acetone (98 vol acetone, 2 vol 1 mol/l HCl) and dried in air.

Precipitation of histones by polyanions. To a series of test-tubes, each containing 0.5 mg of total calf thymus histones in 0.14 mol/l NaCl with 5 mmol/l sodium phosphate buffer of pH 7.2 (buffered saline) polyanions were added (0.05—0.5 mg in buffered saline). The final volume in each tube was made up to 2 ml by adding buffered saline. After 5 min at 4 °C the tubes were centrifuged ($2500 \times g$; 15 min) and the absorbance of the supernatant at 230 nm was measured. Concentrations of histones were calculated using $A_{230} = 3.3$ for 1 mg histone/ml (Hnilica 1975).

Interaction of histones with polypentosesulphate determined by the extraction with 0.25 ml/l HCl: The histones dissolved in buffered saline were precipitated with polypentosesulphate as described above. After centrifugation, the pellets were rewashed in 1 ml buffered saline, centrifuged and both supernatants combined (fractions B — free histones). The pellets were then extracted twice in 0.25 mol/l HCl and centrifuged to separate supernatants (fractions C — histones extracted with HCl) and pellets (fractions A — histones not extracted with HCl). The supernatants B and C were precipitated overnight in 2 mol/l TCA at −20 °C. The precipitates of B, C as well as fraction A were washed with acidified acetone, dried in air, redissolved in SDS sample buffer and electrophoresed as described below.

Effect of NaCl on the solubility of polyanion-histone complexes: Polyanion-histone complexes (prepared at a ratio polyanion/histone = 0.4) — insoluble in buffered saline — were prepared as outlined above. The amount of histones bound with polyanion was calculated from the difference between the absorbance at 230 nm of the histone solution before and after the addition of the

polyanion. The pellets were then mixed with 2 ml NaCl solutions of different concentrations. After thorough vortexing each tube was spun at 2500 × *g* for 15 min and the absorbance of the supernatant at 230 nm was measured. The absorbance of polyanions at 230 nm was negligible.

Separation of ^{3}H-polypentosesulphate-histone complex in 2 mol/l NaCl on a Sephadex column: The histones precipitated with ^{3}H-polypentosesulphate (at a ratio polyanion/histone = 0.4) as described above were solubilised in 2 mol/l NaCl buffered by 5 mmol/l Tris. HCl of pH 7.2. This sample of disolved ^{3}H-polypentosesulphate-histone complex was loaded onto a gel column (17 × 370 mm) containing 1 : 1 mixture of Sephadex G-75 and G-100. The column was preequilibrated and eluted with 2 mol/l NaCl, 5 mmol/l Tris. HCl, pH 7.2 solution. In each fraction (2.7 ml) the absorbance at 230 nm and radioactivity were measured. One ml of each fraction was precipitated with 2 mol/l TCA at −20 °C overnight, washed twice with acidified acetone and the precipitate was redissolved in SDS sample buffer and put on 15 % polyacrylamide gel slab.

Effect of pH on the solubilisation of polyanion-histone complexes: To the pellets of histones precipitated with polyanions (at a ratio polyanion/histone = 0.4) as described earlier, 2 ml of 0.14 mol/l NaCl containing 5 × diluted Britton—Robinson buffer of pH 7—12 was added. After thorough stirring each tube was centrifuged at 2500 × *g* for 15 min and the absorbance of the supernatant at 230 nm was measured.

Polyacrylamide gel electrophoresis: Samples for electrophoresis were dissolved in sample buffer and boiled for 1 min prior to loading onto a 15% polyacrylamide sodium dodecyl sulphate slab gels prepared by the procedure of Laemmli and Favre (1973). Electrophoresis was performed at 40 mA for 6 hours, gels were then stained for 20 min at 80 °C in Coomassie blue *R*-250 (50 % methanol and 10 % acetic acid) and destained in 5 % methanol and 10 % acetic acid overnight.

Liquid scintillation counting: Samples (0.2 ml) were counted in a dioxan-based scintillation liquid; for samples containing NaCl solution, measurements were carried out only 2 hours after mixing.

Results

Precipitation of free histones with polyanions: As demonstrated in Figure 1 histones precipitate in 0.14 mol/l NaCl with all applied polyanions. The maximum of precipitation by polyanions was reached at the ratio polyanion /histone = 0.2—0.3. In this range of values more than 90 percent of histones form insoluble complexes with all polyanions used. After the addition of higher amounts of polyanions (the total volume being kept constant) the curves turn up and a still larger portion of histones remains unprecipitated in the solution. Thus, at a ratio polyanion/histone = 1 the complexes of histones with dextran sulphate and copolymer ethylene-maleic acid were mostly soluble; the complexes of histones with heparin and polypentosesulphate were only partially soluble. Inorganic polyphosphate, even at higher concentrations, is, however, too "weak" to solubilise the formed complex.

It is generally known that histones are readily extractable from the chromatin complex in strong acids (0.25 mol/l HCl or 0.2 mol/l H_2SO_4). However, Berlowitz et al. (1972) pointed out that histones could not be extracted from the complex

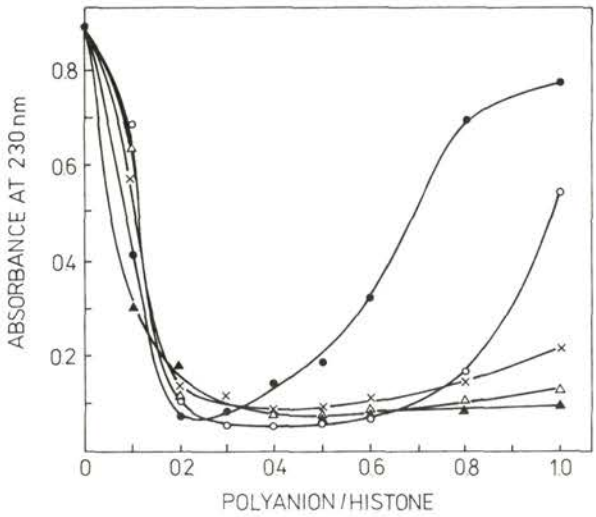

Fig. 1. Precipitation of free histones with polyanions in buffered saline. The coordinate shows the amounts of histones remaining in supernatant after centrifugation of mixture of whole histone with increasing amounts of polyanions in buffered saline (measured as absorbance of the supernatant at 230 nm). △, polypentosesulphate; × heparin; ○ dextran sulphate; ▲, inorganic polyphosphate; ●, copolymer ethylene-maleic acid.

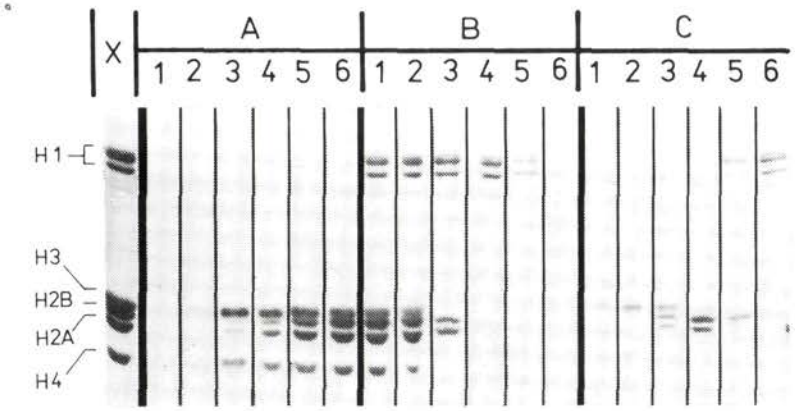

Fig. 2. Polyacrylamide gel electrophoresis of histones after reaction with polypentosesulphate in 0.14 mol/l NaCl solution. (*A*), histones precipitated with polypentosesulphate but not extracted by 0.25 mol/l HCl; (*B*), unprecipitated histones remaining in supernatant; (*C*), histones precipitated with polypentosesulphate but extracted by 0.25 mol/l HCl. The ratios of polypentosesulphate/histone were (1), 0.02; (2), 0.05; (3), 0.1; (4), 0.15; (5), 0.2; (6), 0.4.

with polystyrene sulphonate by 0.2 mol/l H_2SO_4. All precipitated complexes of histones with polyanions used in this study proved to be insoluble in 0.25 mol/l HCl.

Interaction of histones with polypentosesulphate determined by the extraction of histones with 0.25 mol/l HCl: We have found that complexes of histones with all polyanions used can be solubilised and dissociated in 2 % SDS which is present in the sample buffer for electrophoresis according to the method of Laemmli and Favre (1973). This finding helped us to determine the order of binding of individual histone fractions with polyanions. As representative example, the polypentosesulphate-histone complex was studied.

The results are summarized in Figure 2. With the use of increasing amounts of polyanion still more histones appear (at first H3 and H4, followed by H2A and H2B) in the fraction A (Figure 2*A*), i.e. they cannot be extracted from the complex by acid. A small proportion of the histones precipitated by polypentosesulphate (Fig. 2*C*) is, however, still extractable with 0,25 mol/l HCl. Under the conditions used (at a ratio polypentosesulphate/histone = 0.4) histone H1, disappearing from the supernatant (Figure 2*B*, slot 6), i.e. precipitated in 0.14 mol/l NaCl with polypentosesulphate, can be completely extracted in 0.25 mol/l HCl (Fig. 2*C*, slot 6). However, after a longer time of precipitation all histone fractions, including H1, became completely unextractable with 0.25 mol/l HCl (not shown). Affinities of the histones for polypentosesulphate appeared in the order from greatest to least: H4 ~ H3 > H2A > H2B > H1.

Stability of polyanion-histone complexes: It is well established that histones bound with DNA in chromatin or reconstituted deoxyribonucleohistone dissociate from the complex at alkaline pH or at higher ionic strength. In regard to the polyanionic character of DNA, similar behaviour of complexes formed between natural or synthetic polyanions with histones is to be expected.

(a) *Influence of pH*: We studied the effect of pH on the behaviour of polyanion-histone complexes in the range of pH 7—12 (the complexes prepared were fully insoluble below pH 7).

Complexes of histones with polyanions were mixed with 0.14 mol/l NaCl containing 5 × diluted Britton—Robinson buffer of different pH. The amounts of histones released into the solution were estimated from the absorbance of the supernatant at 230 nm (Figure 3). The complexes prepared were stable up to almost pH 10. A further increase of pH caused a "sudden" solubilisation of histones. The solubilisation of the complexes, as measured at pH 11, increased in the order: polypentosesulphate > heparin > copolymer ethylene-maleic acid >

Discussion

The addition of polyanions (heparin, polypentosesulphate, dextran sulphate, polyphosphate or copolymer ethylene-maleic acid) to the solution of histones in buffered saline results in precipitation of the proteins. Polyanions cause a maximal precipitation of histones at a polyanion/histone ratio equal to 0.2—0.3. However, when higher amounts of polyanions were added (up to a ratio polyanion/histone = 1) a still larger proportion of histones remains soluble; the histone complexes with dextran sulphate and copolymer ethylene-maleic acid do not precipitate appreciably at this ratio. Similar results for heparin and dextran sulphate were described previously by Kent et al. (1953). The solubility of the polyanion-histone complexes at higher amounts of polyanions is probably due to the binding of excessive polyanion molecules to the complexes which then gain additional negatively charged groups and become soluble. The solubility of polyanion-histone complexes at higher amounts of sulphate groups containing polyanions increases with the molecular weight of the polyanions.

Experiments on the interaction of polypentosesulphate with isolated histones indicate that the four histones H4, H3, H2A and H2B have a greater affinity for polypentosesulphate than the histone H1 (H3 ~ H4 > H2A > H2B > H1). Similar results were also reported for polyphosphate (Paponov et al. 1978), polystyrene sulphonate (Berlowitz et al. 1972) and heparin (Hildebrand et al. 1977). The order of affinity of histones to polyanions is thus inverse to the order in which the various histone fractions are known to be extracted from chromatin by increasing concentrations of NaCl or HCl. Only Kitzis et al. (1976) reported that H2A and H2B histones were the first in the order of histone fractions which bind with heparin.

Although the H1 histone has a higher positive charge than single H4 and H3 molecules, it is last in the order of binding with polyanions. The existence of $(H3)_2(H4)_2$ complexes in aqueous solutions (Roark et al. 1976) may account for the higher affinity for polyanions as compared with the histone H1 which is unable to aggregate (Roark et al. 1976; Sperling and Bustin 1976).

Histones are readily soluble in strong acids such as HCl or H_2SO_4; but become insoluble at acidic pH values in complexes with polyanions (Berlowitz et al. 1972). We have confirmed these observations for all polyanions used. In the case of polypentosesulphate we have found that a small amount of histones, precipitated with polyanion polypentosesulphate for a short time (5 min), remains extractable in 0.25 mol/l HCl. This may be explained by the possibility that some histone molecules are not yet fully saturated with polyanions in the first stage. However, after a longer time of precipitation, stronger binding of polypentosesulphate with histone (probably due to full saturation) was observed, as judged from a failure to extract all precipitated histone fractions by 0.25 mol/l HCl.

Miller et al. (1971) and Berlowitz et al. (1972) reported that a complex of

polystyrene sulphonate with histones was soluble in an alkaline solution of pH 9. However, in our experiments all polyanion-histone complexes (used in this study) were completely soluble only at pH 12.

The insolubility of the polystyrene sulphonate-histone complex in 5 mol/l NaCl or in 10 mol/l urea (Miller et al. 1972) led the authors to conclude that the complex was not ionic in nature. We have, however, demonstrated a complete solubilisation in 2 mol/l NaCl at pH 7.2 of all polyanion-histone complexes used in this study. Moreover, the solubility of the polypentosesulphate-histone complex in 2 mol/l NaCl proved to be due to its dissociation. These results clearly demonstrate the ionic nature of the latter complex. We suppose that the dissociation of other studied polyanion-histone complexes can also be accounted for by their solubilisation in 2 mol/l NaCl. Recently Courvalin et al. (1982) described a dissociation of the heparin-histone complex (prepared by the action of heparin on chromatin) in a solution containing 0.35 mol/l guanidium chloride and 6 mol/l urea.

We suppose that different molecular weights of polypentosesulphate (2000) and polystyrene sulphonate (18,000), as well as their different chemical structures and charge densities, may account for the differences in the behaviour of their complexes with histones as found by ourselves for polypentosesulphate, and by other authors (Berlowitz et al. 1972; Miller et al. 1971; Miller et al. 1972) for polystyrene sulphonate.

As outlined above, the interaction of free histones with synthetic polyanion polypentosesulphate is of an ionic nature as complex formation can be reversed (at least in a solution of 2 mol/l NaCl). In vivo, factors other than higher ionic strength probably have to be accounted for the release of histones from their binding to polyanions (e.g. cellular polycations could be able to pull down polyanions from histones). These and similar questions concerning the mutual interaction of chromatin or histones with polyanions (e.g., the formation of chromatin-polyanion complex — Courvalin et al. 1982; the effect of polyanions on chromatin accessibility to enzymes — Furukawa and Bhavanandan 1982; and the changes in nucleosomal arrangement caused by polyanions — Doenecke 1980, 1981) are still at the centre of studies in several laboratories. We are of the opinion that the interaction of histones with polyanions offers a useful model for understanding the physico-chemical basis of the interaction of nuclear chromatin histones with natural polyanions, which will be the subject of our future research.

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