Pitfalls in the use of carboxylic acid anhydrides for structural studies of nucleoprotein particles

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During modification of protein amino groups with carboxylic acid anhydrides, these reagents cause a fall in pH, which can be prevented by addition of base. Although unmodified nucleosomal particles are not affected by the local transient changes in pH induced by the base (NaOH) added to prevent a fall in pH during modification, the nucleosomal particles modified by acetic anhydride are dissociated, with release of single-stranded DNA.

INTRODUCTION

Carboxylic acid anhydrides modify protein amino groups in aqueous solution, with a large amount of added reagent being hydrolysed [1]. Since both reactions are accompanied by release of proteins, a fall in pH in the modification mixture is produced, which can be avoided by addition of base. The maintenance of a basic pH during reaction is especially important with reagents such as monomethyl- and dimethyl-maleic anhydrides, the reaction products of which are unstable at neutral and acid pH, with regeneration of the amino groups [2]. This reversibility and the electrostatic destabilization introduced make these anhydrides convenient reagents to dissociate protein-containing structures, allowing the subsequent recovery of the unmodified dissociated components upon regeneration of the modified amino groups [3–5]. However, since low and high pH are known to disrupt base-pairing in free nucleic acids, disturbing effects on the nucleoprotein particles being modified might be produced by the local transient changes in pH that accompany the base addition used to keep the pH constant throughout modification. The present paper shows that, in spite of efforts to diminish sudden changes in pH upon base addition, previous conditions used for modification of nucleosomal particles [5,6] cause pH-dependent structural effects.

MATERIALS AND METHODS

Preparation of nucleosomal particles

Nuclei were obtained from chicken erythrocytes after lysis of the cells in a buffer solution containing 0.5% of the non-ionic detergent Nonidet P40 [7]. The isolated nuclei were digested with micrococcal nuclease (Cooper), and extracted with 0.25 mM-EDTA (pH 8.0) [8]. Nucleosomal particles containing histones H1 and H5 were precipitated with 100 mM-KCl/0.25 mM-EDTA (pH 8.0), and mononucleosomal particles lacking histones H1 and H5 were isolated by centrifugation on a 5–20% sucrose gradient as described below. The concentration of nucleosomal particles was determined spectrophotometrically, taking $A_{260} = 20.0$ for a solution containing 1.0 mg of DNA/ml.

Treatment of nucleosomal particles with acetic anhydride and acetic acid

Nucleosomal particles (0.1 mg of DNA) in 1 ml of 10 mM-Hepes (pH 8.2)/5 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride were treated at room temperature with either 1 mg of acetic anhydride in dioxan (250 mg/ml) or 1 mg of acetic acid in water (100 mg/ml). Both solutions were added in 2 μ l portions. During treatment, the pH was maintained at 8.2 by addition of 1 M-NaOH (2 μ l portions). Modification was also performed without pH adjustment by addition of base. In this case, the nucleosomal particles were modified in 100 mM-Hepes (pH 7.5)/0.1 mM-phenylmethanesulphonyl fluoride. At the end of modification the pH had fallen to 7.2. After all treatments the preparations were dialysed overnight at 2–4 °C against 10 mM-Tris/HCl (pH 8.2)/5 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride.

Sedimentation analysis and fractionation

Preparations were centrifuged in linear 5–20% sucrose gradients containing 10 mM-Tris/HCl (pH 8.2), 5 mM-EDTA and 0.1 mM-phenylmethanesulphonyl fluoride, in a Beckman SW40 rotor at 30000 rev./min for 26 h at 6 °C. The distribution along the gradient of material absorbing at 254 nm was determined with an ISCO density fractionator, which was also used for the separation of fractions.

Elution from hydroxyapatite

The column of hydroxyapatite (300 mg in 150 mM-sodium phosphate, pH 6.9) was prepared in a plastic syringe (1 cm inner diameter), the narrow end of which had been plugged with cheesecloth. Elution took place by centrifugation at 2000 g for 10 min. DNA was purified from the different fractions, after treatment with proteinase K, by extraction with phenol/chloroform, and precipitation with ethanol [9]. The DNA sample was added in 1 ml of 150 mM-sodium phosphate (pH 6.9). The eluted fraction and that obtained by a subsequent washing with 0.35 ml of the same buffer solution were mixed to form the fraction of single-stranded DNA. The double-stranded DNA fraction was eluted in two steps

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Fig. 1. Sedimentation patterns of differently treated nucleosomal particles

Nucleosomal particles: (a) untreated; (b) treated with acetic anhydride in 10 mM-Hepes (pH 8.2)/5 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride, with addition of 1 M-NaOH to avoid the fall in pH; (c) treated with acetic anhydride in 100 mM-Hepes (pH 7.5)/0.1 mM-phenylmethanesulphonyl fluoride, without base addition; (d) treated with acetic acid and neutralized with 1 M-NaOH; (e) treated with acetic anhydride in 100 mM-Hepes (pH 7.5)/0.1 mM-phenylmethanesulphonyl fluoride, without base addition, and subsequently with acetic acid and 1 M-NaOH. The arrows indicate the position of the untreated nucleosomal particles.

with 1 ml and 0.35 ml of 500 mM-sodium phosphate (pH 6.9).

RESULTS AND DISCUSSION

As reported previously [6], treatment of nucleosomal particles with acetic anhydride, with maintenance of the pH constant during reaction by addition of base (KOH or NaOH), is accompanied by dissociation from the particles of single-stranded DNA (Fig. 1b, Table 1). This released DNA was also characterized as single-stranded by digestion with nuclease S_1 and by thermal denaturation (see reference [6]). In addition, no histones were detected in the corresponding fraction. The pH effect takes place in spite of the gradual addition of base, either manually or with a Radiometer (Copenhagen, Denmark) titration assembly, to the modification mixture, which is continuously stirred by a magnetic bar. Although the pH was monitored continuously during modification, no transient changes larger than ± 0.2 pH unit were detected. However, if the reagent treatment is performed without base addition, in the presence of a higher buffer concentration [100 mM-Hepes (pH 7.5)/0.1 mM-phenylmethanesulphonyl fluoride], no dissociation of DNA is observed (Fig. 1c), and the DNA present in the particles remains double-stranded (Table 1).

To find out whether the release of single-stranded DNA is only the result of the local pH changes brought about by base addition, the nucleosomal particles were treated with an amount of acetic acid equivalent to that of acetic anhydride used for modification, and the pH was kept constant by addition of base. Fig. 1(d) shows that no dissociation of DNA takes place under these conditions, which indicates that the addition of base causes no change in unmodified nucleosomes, and that DNA release and denaturation are not merely the effects

Table 1. Elution from hydroxyapatite of different DNA preparations

The preparations and fractions of nucleosomal particles are the same as in Fig. 1. DNA was evaluated from the A_{260} , by considering a hyperchromicity of 30% for the DNA not retained by hydroxyapatite. The recovery of DNA from the column was higher than 90%.

DNA preparation	DNA not retained by hydroxyapatite (%)
a	10
a (DNA heated)*	90
b, Fraction I	10
b, Fraction II	90
c	10
d	10
e, Fraction I	12
e, Fraction II	84

* Denatured by boiling for 5 min.

of local pH changes, but that modification is also required. When nucleosomal particles, previously modified by acetic anhydride without base addition, are subjected to treatment with acetic acid and base, dissociation of single-stranded DNA takes place (Fig. 1e, Table 1). As with the nucleosomal particles modified with addition of base, no histones were detected in the fraction of released DNA. These results indicate that the modified nucleosomal particles are more susceptible than the original nucleosomes to DNA release and denaturation induced by changes in pH.

As shown in this work, modification of nucleoprotein particles by carboxylic acid anhydrides with the pH kept constant by addition of a strong base (NaOH or KOH)

[5,6,10,11,12] is a questionable experimental procedure. In particular, the conclusion drawn by us previously [6], that the weakening of histone-DNA interaction by modification of the lysine residues of particles is accompanied by release of single-stranded DNA, is not correct. In the work by Wallace *et al.* [12], the unidentified slow-sedimenting component which appears in the preparation of nucleosomes treated with acetic anhydride (7 mM) is very likely single-stranded DNA released by the NaOH addition. Although no dissociation to single-stranded DNA is found when modification of lysine residues is conducted without base addition, at moderate ionic strength (0.25 M-NaCl) modification by either acetic or dimethylmaleic anhydride is accompanied by dissociation of the nucleosomal particles and release of double-stranded DNA (M. A. Nieto & E. Palacián, unpublished work). These results explain the previously observed release of double-stranded DNA, when modification by acetic anhydride takes place in 0.6 M-NaCl and the pH is adjusted during reaction by addition of KOH [6]. On the other hand, the same reported biphasic release of histones H2A and H2B by modification of nucleosomal particles with dimethylmaleic anhydride [5] is also observed in the absence of the transient changes in local pH induced by base addition (M. A. Nieto & E. Palacián, unpublished work).

In conclusion, neutralization with strong bases should be avoided when using carboxylic acid anhydrides to modify nucleoprotein particles.

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