Cross-linking of histones with dimethyl 3,3'-dithiobispropionimide

Interference by a one-end reaction modifying histones at lysine amino groups

Evert KOTTHAUS and Wolf H. STRÄTLING*

*Physiologisch-Chemisches Institut, Universitäts-Krankenhaus Eppendorf, Martinistrasse 52,
D-2000 Hamburg 20, Federal Republic of Germany*

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We have studied the HClO_4 -solubility of histones H1 and H5 in hen erythrocyte nuclei after treatment with the cross-linker dimethyl 3,3'-dithiobispropionimide (DTPI). The amount of acid-soluble, non-cross-linked, H1 and H5 histones was drastically decreased, and that of acid-soluble H1/H5 histone dimers went through an optimum as the DTPI concentration was raised. Incubation of the HClO_4 -insoluble fraction with 2-mercaptoethanol regenerated the acid-solubility of H1/H5 histones in this fraction. When purified H1/H5 histones were treated with increasing concentrations of DTPI under non-cross-linking conditions, the amount of HClO_4 -soluble histones also greatly decreased, but to a much lesser extent if the DTPI treatment was followed by reduction with 2-mercaptoethanol. This decrease was inversely correlated to the proportion of amino groups modified. It is concluded that, when the cross-linker was used in large excess, the cross-linking reaction competed with a one-end reaction modifying the histones at lysine amino groups by cross-linker molecules, of which the imidoester groups that had not reacted were hydrolysed. It is suggested that this modification produced the changes in acid-solubility.

Bisimidoesters have been frequently used to elucidate the nearest-neighbour relationship of histones in chromatin (Thomas & Kornberg, 1975; Thomas & Khabaza, 1980; Wyns *et al.*, 1978; Dashkevich *et al.*, 1983). In particular, cleavable reagents, such as DTPI (Wang & Richards, 1975), offer great advantages for the identification of the cross-linked proteins. However, at least two studies indicate that their use also creates some problems. Ring & Cole (1979) reported that, after treatment of steer kidney nuclei with DTPI, the recovery of lysine-rich histones during subsequent extraction with HClO_4 decreased drastically as the cross-linker concentration was raised. Thomas & Khabaza (1980) observed that, after treatment of rat liver chromatin with dimethyl suberimide, the recovery of lysine-rich histone oligomers during HClO_4 extraction first increased and then decreased in a time-course experiment. The causes for these acid-solubility changes are not known, but they seem to indicate gross changes in the electrostatic character of the proteins treated with

bisimidoesters. It is likely that the histones, owing to their basic character and owing to the use of acid extraction after cross-linking, represent only a very drastic case for a problem inherent in all studies involving the use of bisimidoesters. We therefore started a more careful study with chicken erythrocyte nuclei and purified lysine-rich histones (H1 and H5) as a protein source and DTPI as a cross-linker. We conclude that the observed changes in acid-solubility were produced by one-end reactions modifying the histones at lysine amino groups by cross-linker molecules, of which the imidoester groups that had not reacted were hydrolysed.

Materials and methods

Treatment of hen erythrocyte nuclei with DTPI at the indicated concentrations at pH 8.2 was performed as described elsewhere (Kotthaus & Strätling, 1984). The nuclei suspensions were then made 5% (w/v) with respect to HClO_4 and stirred for 30 min at room temperature. Subsequent centrifugation at 10000g separated each sample into a supernatant (HClO_4 -soluble fraction) and a pellet (HClO_4 -insoluble fraction). The proteins in

Abbreviation used: DTPI, dimethyl 3,3'-dithiobispropionimide.

* To whom correspondence should be addressed.

the supernatants were precipitated with trichloroacetic acid at 18% (w/v), collected by centrifugation and dissolved in sample buffer [6M-urea/2% (w/v) sodium dodecyl sulphate/62mM-Tris/HCl, pH6.8]. The HClO_4 -insoluble fractions were re-extracted twice with 0.2M- H_2SO_4 , dialysed against cross-linking buffer (Kotthaus & Strätling, 1984) and treated for 2h at 40°C with 20% (w/v) 2-mercaptoethanol. The samples were then dialysed against 5% HClO_4 , then centrifuged, and the proteins in the supernatant fraction were precipitated with 18% trichloroacetic acid. Histones were resolved by electrophoresis on 12%-(w/v)-polyacrylamide gels with 3.5% stacking gel in the presence of sodium dodecyl sulphate (Laemmli, 1970). Gels were scanned at 555nm in a Beckman DU-8 spectrophotometer.

H1/H5 histone fraction was prepared by extraction from erythrocyte nuclei with 5% HClO_4 and was treated with DTPI at the indicated concentrations. Fractionation with 5% HClO_4 and treatment of the soluble and insoluble fractions were performed as described for DTPI-treated nuclei, except that the H_2SO_4 extraction was omitted.

Quantification of the number of thiol groups followed the procedure described by Deakin *et al.* (1963). Briefly, samples containing DTPI-treated H1/H5 histone fraction were dialysed against three changes of 20mM-triethanolamine/HCl, pH7.2, and incubated with bis-(2-carboxy-3-nitrophenyl) disulphide at 90mmol/mmol of histone for 3h under N_2 atmosphere. After centrifugation at 12000g, the absorbance of the supernatant was measured at 412nm. The reaction of unmodified H1/H5 histone fraction with bis-(2-carboxy-3-nitrophenyl) disulphide was used as blank. Protein concentrations were determined by the method of Lowry *et al.* (1951), with purified H1/H5 histone fraction as standard.

Results and discussion

Hen erythrocyte nuclei were treated with various concentrations of DTPI and then extracted with 5% HClO_4 . The protein composition in the soluble fractions was displayed by electrophoresis on polyacrylamide gels as shown in Fig. 1(a). HClO_4 selectively extracted non-cross-linked H1 and H5 histones and H1/H5 dimers. A trace amount of H1/H5 trimers was also formed. The pellets remaining after the 5% HClO_4 extraction were re-extracted with 0.2M- H_2SO_4 . Gel electrophoresis showed that the H_2SO_4 extracts contained non-cross-linked core histones, core histone dimers, dimers between H1/H5 histones and core histones, and trace amounts of non-cross-linked H1 and H5 histones (results not shown). The HClO_4 -insoluble fractions were then treated with 2-mercaptoethanol and extracted for a second time with 5% HClO_4 . The histones recovered were resolved by electrophoresis on polyacrylamide gels as shown in Fig. 1(b). Comparison of the gel in Fig. 1(a) with that in Fig. 1(b) showed that the amount of non-cross-linked H1/H5 histones in the HClO_4 -soluble fraction progressively decreased, whereas that of the H1/H5 histone fraction, which remained insoluble during the first HClO_4 extraction but was rendered acid-soluble by 2-mercaptoethanol treatment, increased as the concentration of DTPI was raised. A quantitative evaluation of these data is presented in Fig. 2. The amount of H1/H5 dimer recovered in the HClO_4 -soluble fraction was maximal (about 12% of the original H1/H5 histone fraction) at 1.2mg of DTPI dihydrochloride/mg of histone and then gradually decreased, as the cross-linker concentration was further raised.

Several aspects of these results were surprising. First, when the DTPI concentration was in-

Fig. 1. Treatment of hen erythrocyte nuclei with various concentrations of DTPI

Hen erythrocyte nuclei were treated with 0 (a), 0.2 (b), 0.4 (c), 0.8 (d), 1.2 (e), 1.6 (f), 2.0 (g), 2.5 (h) and 3.0 (i) mg of DTPI dihydrochloride/mg of histone. Histones were then fractionated with 5% HClO_4 into a soluble fraction (a) and an insoluble fraction (b).

Fig. 2. Proportions of H1/H5 dimers in the HClO_4 -soluble fraction (Δ), of non-cross-linked H1/H5 histones in the HClO_4 -soluble fraction ($\diamond$) and of H1/H5 histones in the HClO_4 -insoluble fraction ($\square$)

The sum of H1/H5 histones in the three fractions is also shown ($\circ$).

creased, an increasing portion of the H1/H5 histone fraction was rendered insoluble in HClO_4 . However, reduction with 2-mercaptoethanol regenerated the HClO_4 -solubility. Cross-linking of H1/H5 histones with core histones into heteropolymers or larger homopolymers cannot account for these results, since the former were produced only in very small amounts and the latter were not detected either in the H_2SO_4 extract or in the HClO_4 extract. Secondly, the amount of H1/H5 dimers in the HClO_4 -soluble fraction went through an optimum as the DTPI concentration was raised. This response suggested the occurrence of two processes competing with each other with respect to the yield of H1/H5 dimers. The process increasing the dimer yield represents the cross-linking reaction. In search for a process decreasing the dimer yield and further making a significant contribution to the decreased of the yield of non-cross-linked monomers in the HClO_4 -soluble fraction, we thought of a reaction in which DTPI reacts at only one end. At pH 8.2 hydrolysis of the imidoester group is a very rapid reaction with a half-life for the disappearance of the imidoester of about 2–5 min (Browne & Kent, 1975). Thus one-end reactions might have occurred in two ways. When one imidoester group in a DTPI molecule is hydrolysed, a molecule with only one reactive group is generated. Alternatively, after reaction of one imidoester group with a lysine amino group, the end that has not reacted is hydrolysed. In particular, this latter type of reaction is expected to be increasingly favoured when the DTPI is added at an increasingly large excess. A one-end reaction

replaces an amino group by an amidino group, which retains the positive charge. However, since the major product of hydrolysis is the ester (Browne & Kent, 1975), the amidino group is followed by a hydrophobic chain ($-\text{CH}_2-\text{CH}_2-\text{S}-\text{S}-\text{CH}_2-\text{CH}_2-\text{CO}-\text{OCH}_3$). The amide is likely to be also formed by hydrolysis (Peters & Richards, 1977), thereby generating a chain of similar hydrophobicity. One-end reactions at multiple sites therefore confer a hydrophobic character to the histone. It is suggested that this change in character produced the decrease in acid-solubility. Splitting off the hydrolysed moiety of the cross-linker by cleavage of the disulphide bridge is expected to lower the conferred hydrophobic character and is therefore expected to regenerate, at least partially, the original basic character.

To lend support to the explanation outlined in the preceding paragraph, we treated purified H1/H5 histone fraction with DTPI dihydrochloride at 2.5, 5, 10 and 12.5 mg/mg of histone. Assuming a number of, on the average, approximately 51 amino groups per H1/H5 molecule (see the legend to Fig. 3b), these DTPI concentrations corresponded to a 7-, 14-, 28- and 35-fold respectively excess of imidoester groups over lysine amino groups. Control gels showed that cross-linking did not occur under the conditions used (results not shown). After DTPI treatment the samples were made 5% with respect to HClO_4 . As shown in Fig. 3(a), the yield of HClO_4 -soluble H1/H5 histones drastically decreased when the DTPI concentration was raised. The solubility response in 0.2M- H_2SO_4 was nearly the same as that in 5% HClO_4 (results not shown). When the DTPI-treated samples were incubated with 2-mercaptoethanol before HClO_4 extraction, the loss of H1/H5 histones in the HClO_4 -insoluble fractions was much less (Fig. 3a). Thus the acid-solubility response of purified H1/H5 histones to DTPI treatment and the acid-solubility response of DTPI-treated H1/H5 histones to reduction with 2-mercaptoethanol were qualitatively similar to that of H1/H5 histones in chromatin. Complete removal of the modification by aminolysis nearly restored the original HClO_4 -solubility of H1/H5 histones before the DTPI treatment (Fig. 3a).

After a sequential treatment of H1/H5 histone fraction with DTPI and 2-mercaptoethanol, we determined the average number of thiol groups introduced into each histone molecule. The values obtained are expressed as percentages of amino groups modified. When the DTPI concentration was raised, the significant increase in the proportion of amino groups modified correlated well with the decrease in HClO_4 -solubility (compare Fig. 3a with Fig. 3b). These results thus support the above suggestion that, when bisimidoesters are used in

Fig. 3. Treatment of purified H1/H5 histones with DTPI (a) After treatment of H1/H5 histones with DTPI at the indicated concentrations, the samples were fractionated with 5% HClO₄ without (○) and with (□) prior treatment with 20% 2-mercaptoethanol. ■, Fractionation after prior treatment with 2% NH₃ for 20 h at 23°C. (b) Proportion of amino groups modified by DTPI. After sequential treatment with DTPI and 2-mercaptoethanol, the frequency of thiol groups was estimated (mean $\pm$ s.d. for three determinations) and expressed as percentages of total H1/H5-histone amino groups. The calculation is based on a number of 45 amino groups in H5 histone (Briand *et al.*, 1980) and uses a value of ten amino groups in the sequenced portion (residues 1–51) of chicken erythrocyte H1 histone (CEL-5) and a number of 55 lysine residues in the portion from residue 54 to C-terminus of rabbit thymus H1 histone (RTL-3) (Cole, 1977). Lys-51 of CEL-5 is homologous to Lys-53 of RTL-3 (Cole, 1977). By using further a H1/H5 ratio equal to 0.4:0.9 (Bates & Thomas, 1981), the average molar content of amino groups in H1/H5 histones was calculated to be 51.

large excess, one-end reactions followed by hydrolysis of the imidoester group that had not reacted modified the electrostatic character of the histone

and thereby produced the change in acid-solubility. This explanation is also offered for those studies that reported previously that the yield of H1/H5 dimers goes through an optimum in a concentration-dependent experiment (Ring & Cole, 1979) and in a time-course experiment (Thomas & Khabaza, 1980).

It is possible that the modification of amino groups by hydrophobic chains also affects properties other than the acid-solubility, e.g. the conformation of the protein or protein-protein interactions and thereby the conformation of the oligomeric complex. Since most cross-linking studies aim at the elucidation of the latter properties particularly, changes in these properties are highly undesirable. As a precaution, we suggest the use of bisimidoesters at concentrations as low as possible.

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