The reactivity of the thiol groups of calf thymus deoxyribonucleohistone

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ABSTRACT

The reactivities of the two cysteine thiol groups of calf thymus F3 histone were investigated using 5,5'-dithiobis-[2-nitrobenzoic acid], (DTNB). In isolated histone, both thiol groups were available for reaction. However, analysis of reaction profiles of native deoxyribonucleohistone, (DNH), in various solvent conditions, together with gel electrophoresis studies of DNH modified with DTNB, showed that only one of the thiol groups is normally modified by the reagent. If NaCl is present (above 1.0M) the other thiol group can also be modified. The reactivities of both groups were largely independent of the degree of DNH supercoiling and of the binding of F3 to the DNA.

INTRODUCTION

The nuclei of eukaryotic organisms contain DNA complexed to the very basic histone proteins, RNA, and non-histone or acid-insoluble proteins¹. The histones are thought to be mainly of importance in maintaining the DNA in a coiled-coil conformation, for example the supercoil proposed by Pardon *et al*². It is possible that they are also involved in the regulation of genetic expression³. However, little is yet known about the arrangement of the histones on the DNA, both with respect to their relative spatial distribution along the double helix, and to the conformations adopted by the individual histones while bound to the DNA. That the interaction between the histones and DNA is extremely specific, however, is suggested by the very high evolutionary stability of the primary sequence of the histones, especially that of F2a1⁴ and F3^{5,6}.

Amongst the five major histone fractions, the alanine-rich arginine-rich F3 histone is unique in containing one or more cysteine residues per molecule which, by virtue of their ability to undergo intra- or intermolecular cross-linking by means of disulphide bridges, may be of importance both structurally and functionally. In common with the F3 histones of all higher eukaryotes so far studied, calf

thymus F3 contains two cysteine residues per molecule^{7,8}.

This study was concerned with measuring the relative reactivity towards the specific thiol reagent 5,5'-dithiobis-[2-nitrobenzoic acid], (DTNB), under various solvent conditions, of the thiol groups of calf thymus F3, (a) where F3 formed part of isolated whole histone, (b) where F3 had been newly dissociated, but not isolated from the DNA and (c) where F3 formed part of the native deoxyribonucleohistone complex. In this way we hoped to gain some insight into the stereochemistry of the thiol groups themselves, and of F3 in relation to the compact nucleohistone structure.

EXPERIMENTAL

Preparation and assay of materials

Soluble deoxyribonucleohistone in 0.7mM sodium phosphate buffer, pH 6.8, was prepared by the method of Zubay and Doty⁹. The DNA content of DNH solutions was measured spectrophotometrically by their absorbance at 260 nm, using an extinction coefficient of $7,400 \text{ M}^{-1}$ phosphorus¹⁰. Protein contents of DNH solutions and solutions of whole histone were measured by the Lowry modification¹¹ of the Folin reaction. Histone to DNA weight ratios of DNH preparations all fell within the range 1.26 to 1.40. Whole histone was isolated from DNH by acid extraction¹² and lyophilised from a 1mM HCl solution.

DTNB assay of thiol groups

A 4mM solution of DTNB (Sigma Chemical Co.) dissolved in 0.033M sodium phosphate buffer, pH 8, was used throughout. Aliquots (0.04 ml) of this solution were added to 3ml of test solution (previously centrifuged at 10,000g for ten minutes to clarify the solution) in a 1cm path length capped cuvette. These quantities represent an approximate sixfold excess of reagent over total thiol content in the test solution. The rise in absorption at 412 nm¹³ was monitored on a Unicam SP500 spectrophotometer. Appropriate solvent corrections were made.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out essentially by the method of Panyim and Chalkley¹⁴. The gels (10cm x 0.6cm i.d., 15% acrylamide, 0.1% N,N'-methylene diacrylamide, 2.5M urea, pH 2.7) were pre-electrophoresed for about 16 hours at 1mA per tube. The electrophoresis was carried out at 2mA per tube for 3 hours. Approximately 50µg of protein were loaded on each gel. The gels were stained overnight

in 0.1% naphthalene black, 20% ethanol, 7% acetic acid and destained electrophoretically in 7% acetic acid. The gel patterns were scanned on a Joyce Loebl double beam microdensitometer and the gel traces analysed on a Dupont curve analyser.

RESULTS

a) Reactivity of thiol groups in whole histone

The absorption values at 412 nm after complete reaction with DTNB of five different solutions of whole histone (isolated from different DNH preparations) in 0.7mM sodium phosphate buffer, pH 6.8, were measured to check the validity of two basic assumptions made in subsequent calculations, namely (a) that the molar extinction coefficient of the coloured anion 3-carboxylato-4-nitrothiophenolate (CNT) released by reaction of one thiol group with one molecule of DTNB has a mean value of $11,400 \text{ cm}^{-1}$, as estimated by Robyt *et al*¹⁵, and (b) that the F3 fraction constitutes 19% of the total histone complement¹⁶. Using these values, and a value of 15,324 for the molecular weight of F3⁸, the estimated number of thiol groups reacting per F3 molecule was 1.97 ± 0.14 in good agreement with the number expected from the sequence studies.

The effects of various concentrations of NaCl on the rate of reaction of solutions of isolated whole histone with DTNB were investigated (fig.1). In low ionic strength (0.7mM sodium phosphate) there was almost instantaneous total reaction of the two thiol groups. Here the proteins are almost totally devoid of secondary structure and such exposure of the thiol groups to the solvent environment is to be expected. On adding NaCl, formation of α -helical and other ordered sections occurs (largely complete on reaching an ionic strength of about 0.25)¹⁷ concomitant with an increase in intermolecular aggregation, a phenomenon to which the histones, especially F3, are highly susceptible¹⁸. It is likely that the observed lower rates of reaction with DTNB on moving from 0.7M to 2.0M NaCl were a reflection of this latter effect. Leaving the whole histone in the presence of 2.0M NaCl for a prolonged period (19 hours) before assay diminished the reaction rate still more, suggesting that further aggregation affecting F3 slowly occurred with time.

Fig. 1. The effect of NaCl on the reaction of isolated whole histone with DTNB.

Fig. 2. The effect of NaCl (0.7 M & 1.0 M) on the reaction of DNase with DTNB.

Fig. 3. The effect of NaCl (1.25 M - 2.00 M) on the reaction of DNH with DTNB.

Fig. 4. The effect of 6M urea, treatment with NaOH, and treatment with α -chymotrypsin on the reaction of DNH with DTNB.

b) Reactivity of thiol groups in DNH

A typical reaction profile of one preparation of DNH in 0.7mM sodium phosphate, pH 6.8, is shown in fig. 2. Curves for different preparations tended to the same limit of approximately 50% of the total thiol groups theoretically available, although there were variations between preparations in the rate of attaining this limit. The half-times for the reaction varied from approximately 30-90 minutes. Variations of pH in the range 6.3 to 8.0 were found to have only a slight effect on the rate, the higher pH accelerating slightly the initial stages of the reaction. Clearly any conformational change occurring over this pH range does not involve the reactive thiol groups. Above pH 8.1, protein-CNT disulphide bonds and DTNB itself are cleaved¹⁵.

Figs. 2 and 3 show the effect of increasing concentrations of NaCl over the same range used for whole histone on the reaction of DNH with DTNB. It has been previously established that in 0.7M NaCl, only fraction F1 is dissociated from the DNH, which maintains its native compactness; in 1.0M NaCl, F1, F2a2 and substantial portions of fractions F2b and F3 are dissociated, and supercoiling of the DNH is lost; in 1.25M NaCl only a small amount of F2a1 remains, while in concentrations above this all the histones are completely dissociated^{19,20}. In direct contrast to the results with whole histone, increasing concentrations of NaCl up to 1.75M enhanced the reaction rate of DNH solutions. More important, the 0.7M NaCl samples still tended towards a limit of 50% reaction of the total thiol groups, (although at a greater rate than for DNH in 0.7mM sodium phosphate), while at NaCl concentrations above 1.25M the 100% limit was closely approached, representing complete reaction of both thiol groups with DTNB. DNH in 2.0M NaCl assayed after 19 hours gave exactly the same curve as the same solution assayed immediately after salt addition, again unlike whole histone.

Although rates varied, the 100% limit for samples of DNH in NaCl concentrations of 1.5M and over was very reproducible from one DNH preparation to another. This was less true for 0.7mM phosphate, 0.7M NaCl and 1.0M NaCl samples where we have on occasion observed upper limits as low as 30%. For any one DNH preparation in all three salt solutions, however, the limit was the same. Moreover, the reaction limit for these salt conditions was never significantly greater than 50%, a value of 52% being the highest observed.

Fig. 4 shows the effect of two different denaturing conditions on the reaction of DNH with DTNB. 6M urea denatures protein secondary structure by disruption of hydrogen bonding and hydrophobic interactions without causing dissociation of histone from the DNA, but the rigid supercoiling is lost²¹. Treatment of DNH with NaOH (the solution was taken to pH 12.5 and then back to neutrality before assay) disrupts the DNA double helix and thus probably the supercoiling¹², but the effect on histone secondary structure is not known. Also shown in Fig. 4 is the curve obtained for DNH that had been treated for one hour prior to the assay with α -chymotrypsin (Worthington Biochemical Corporation Ltd.) using a 30:1 total histone to chymotrypsin weight ratio. This cleaves the F3 molecule at certain points causing 'unspringing' of the supercoil to an extent which results in a structure intermediate between that of the long flexible DNA molecule and the rigid compact DNH complex, although it is not known which of the fragments dissociate from the DNA²².

All the above samples tended towards a limit of 50% of the total thiol groups available, but the urea- and alkali-treated samples reached this limit faster than the native or chymotrypsin-treated samples, whose reactivity did not differ greatly. 6M urea samples showed quite a range of variability between preparations from different calf thymus glands, but unlike the variability shown by different preparations in 1.0M NaCl, the variable extents of reaction generally led to limits greater than 50%. DNH in the presence of 50% ethylene glycol, which primarily disrupts hydrophobic interactions, displayed no reactivity at all towards DTNB.

c) Gel electrophoresis of DTNB-treated DNH

To assess whether the 50% reaction maximum usually obtained for native DNH on 0.7mM sodium phosphate with DTNB represented, as seemed likely, complete modification of only one of the two thiol groups on all the F3 molecules, whole histone was isolated by 0.3M HCl extraction from a sample of native DNH that had been treated with DTNB for a length of time sufficient to give this maximum (about 6 hours in this case). Separate experiments established that DTNB is totally unreactive towards thiol groups at pH values below 3.2, so the excess DTNB present during the acid extraction of the histone did not have any further effect on F3.

The histones were electrophoresed on polyacrylamide/urea gels for 3 hours. A control sample of whole histone isolated from untreated DNH was electrophoresed simultaneously. The F3 band, clearly visible in the latter gel, was evidently moving with a different mobility in the gel of the DTNB treated sample. Analysis of the gel traces (normalised with respect to the areas of the F2a1 peaks) with a Curve Analyser showed that an amount of material (19% of the total) on the gel of the DTNB treated sample corresponding closely to that of F3 on the control gel (20.5% of the total) was now quantitatively electrophoresing very close to F2b, the other bands being unaffected (fig. 5). We thus conclude that the 50% reaction limit of native DNH with DTNB represents an all-or-nothing effect in that only one of the two thiol groups of all the F3 molecules are modified, rather than random amounts of F3 being partially modified, totally modified, or remaining unmodified.

DISCUSSION

In the case of whole histone that has been acid-extracted from DNH, lyophilised and subsequently dissolved in various NaCl solutions, both thiol groups of F3 are available for reaction with DTNB, but the rate of reaction is diminished as the NaCl concentration is raised. (NaCl itself has no effect on the reaction of DTNB with L-cysteine.) It is most likely that over the range of NaCl concentrations used here (0.7 - 2.0M), increasing protection of the thiol groups is caused by increasing intermolecular aggregation of the F3 molecules, either with themselves or with other histone fractions, rather than any further increases in secondary structure content in the protein, which will be largely complete on moving from 0.7mM sodium phosphate to 0.7M NaCl.

When F3 forms part of the native DNH complex however, one of the thiol groups is completely unavailable for reaction, the other reacting quantitatively with the DTNB, as shown by the gel electrophoresis studies. Only when all the histone fractions are dissociated from the DNA in 1.25 - 1.50M NaCl do both thiol groups become exposed, showing maximum reactivity in 1.75M NaCl, in complete contrast to the behaviour of lyophilised F3 histone. Even in the presence of 1.0M NaCl, where about 70% of the F3 fraction has dissociated from the DNA, the non-reactive group has not been deprotected. This suggests firstly that the conformation of lyophilised F3 redissolved in solutions of various NaCl concentrations, and of F3 freshly dissociated from

Fig. 5. Microdensitometer traces of polyacrylamide / urea gels of
 (a) whole histone isolated from untreated DNH and
 (b) whole histone isolated from DTNB-treated DNH.

----- indicates the contribution of the F3 bands as resolved
 by the Curve Analyser, with area relative to total.

DNA in situ by the same NaCl concentrations are quite different, at least in the region of the molecule toward the -COOH terminal end where the two cysteine residues are found. This is supported by the difference in time-dependent aggregation effects observed for whole histone and DNH in 2.0M NaCl. Secondly, it appears that the opening up of the second thiol group in 1.25M or higher NaCl concentrations is due to an intrinsic conformational change in F3, it being coincidental that this point corresponds approximately to the dissociation of all the F3 from the DNA. A reduction in the rate of reaction of DNH with DTNB, due presumably to aggregation effects similar to those observed for whole histone in NaCl, is not apparent until the NaCl concentration is higher than 1.75M.

Treatment of DNH with NaOH causing denaturation of the DNA double helix and disruption of supercoiling leads to greater reactivity of the exposed thiol group but does not expose the buried one. However, mere disruption of the supercoiling alone does not seem to be necessarily the direct cause of the enhanced reactivity, in the light of the fact that such disruption by chymotryptic digestion of the histones does not have any marked effect on the reaction rate. Again, we appear to be observing an effect brought about by changes in protein secondary structure.

Disruption of a certain amount of hydrogen bonding and hydrophobic interactions without protein dissociation by 6M urea also enhances the rate of reaction of the exposed thiol group with DTNB and, to a degree varying between DNH preparations from different calf thymus glands, exposes the buried thiol group, generally to only a slight extent. The effect of ethylene glycol in suppressing totally the reaction with DTNB suggests that subtle conformational changes around the appropriate region of F3 can have a marked effect on the thiol-group reactivity. It is possible that ethylene glycol is inducing formation of new secondary structures that are unstable in an aqueous environment.

Considering the primary structure of the F3 molecule, we find that Cys-96 in the sequence of DeLange et al⁸ lies in a non-basic region rich in hydrophobic residues, i.e. residues 85-105. Construction of a 'helical wheel' of these residues after the manner of Shiffer and Edmundson,²³ or application of Prothero's rules²⁴ indicate that

this region has a very high potential for α -helical formation, much greater than that of the region around the other cysteine residue at position 110 which contains a number of helix destabilising residues.

From the results presented above, we would tentatively identify Cys-110 as possessing the more reactive thiol group, which is exposed in the native DNH complex and which reacts at varying rates depending upon the solvent conditions and which is sensitive to a certain extent to the degree of disruption of protein secondary structure. Cys-96 is thus identified as possessing the normally unreactive thiol group which is strongly protected by secondary structure formation in which it is intimately involved, and which is not effectively destroyed even by 6M urea but undergoes some type of conformational change in high NaCl concentrations which exposes the thiol group.

Moreover, the reactivities of both Cys-96 and Cys-110 are seemingly not dependent on the binding of F3 to DNA in the DNH complex nor on the integrity or otherwise of the supercoiling of the complex. This in turn, in conjunction with the electrophoresis experiment, suggests that F3 is bound to the DNA in such a manner that the region extending approximately from residues 80-120 lies on the outside surface of the DNH supercoil, where Cys-110 is available for chemical modification. This is consistent with studies that have shown the chymotryptic points of attack of F3 in native DNH to be readily accessible, in contrast to those of F2a1 and F2a2, thought to be buried within the supercoil²¹.

Marzluff *et al*²⁵ have suggested that there may be two forms of calf thymus F3, a minority fraction (20%) possessing only one cysteine residue, although other workers^{8,6} have found no evidence to support this. Depending upon whether thiol groups in the minority fraction were exposed or buried, the limit of reaction for native DNH with DTNB would lie between 44% and 55% of the theoretical maximum. It is not possible from our results to differentiate between these possibilities although such heterogeneity might explain deviations from the usual limits observed with certain preparations.

The possession by F3 of only one reactive thiol group in native DNH opens up the possibility of specific spin-labelling of the molecule for e.s.r. studies or of attaching a fluorescence label. At a simple level this could be useful for monitoring conformational changes of F3 while still attached to DNA. Such experiments are in

progress. E.s.r. studies have as yet been made only on non-specifically labelled isolated F3. It is of interest to note that the results suggested that Cys-96 is shielded from the solvent environment by secondary structure formation²⁶.

From a biological point of view, our results deem it unlikely that the thiol groups of F3 are involved in intermolecular disulphide bridge formation as a mechanism for the condensation of active chromatin to inactive chromatin since although up to 80% of the chromatin in calf thymus nuclei is in the condensed state,²⁷ all the F3 molecules have one free thiol group, the other becoming reactive under appropriate salt conditions that would not affect disulphide linkages. This however does not exclude involvement of the thiol groups in biological activity at metaphase²⁸. Indeed it is likely that Cys-110 with its exposed thiol group represents a functional residue both in organisms with two cysteine residues per F3 molecule, and in lower organisms which possess only one, while in the former class of organisms, Cys-96 with its protected thiol group represents a non-functional, non-lethal point mutation of the serine residue found at the equivalent position in, for example, carp testes F3⁵, chicken erythrocyte F3²⁹ and in the minority form (40%) of the heterogeneous pea embryo F3⁶. If our conclusions are correct, then the non-reactivity of the thiol group of Cys-96 results from a high degree of protection by secondary structure and this would explain why such a mutation shows such an unexpected lack of effect on possible biological activity⁷.

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