CREVICES CONTAINING CYSTEINE IN THE TERTIARY STRUCTURE OF CALF THYMUS HISTONE F3*

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Received 24 July 1972

1. Introduction

Recently, the primary structures of most histones have been worked out [1–3] and several significant features have become apparent. The most general are the presence of clustered basic residues at the ends of the histone molecules and the presence of long intermediate regions with a composition similar to that found in globular proteins. From these features it has been suggested [4] that the most hydrophobic regions fold giving rise to conformations capable of protein–protein interactions, whereas the highly positive regions of histones are capable of interacting with DNA, and it is probable that histones are involved in higher levels of structural organization. The statement that histones may have particular conformations is partially supported by several studies which includes ORD [5–7], NMR [4, 8, 9] and fluorescence spectroscopy combined with circular dichroism [10].

Structural information can also be obtained from the electron paramagnetic resonance (EPR) spectra of "spin labels" that are attached to proteins [11–13]. This is the case if the free radical becomes immobilized in space due to steric hindrance. Immobilization of the spin label can occur if the protein reacting group is situated in a protected region. By using an appropriate spin label, valuable information can be obtained about the presence of tertiary structure, as well as about particular conformational changes. In the present study we have chosen a synthetic organic free radical which is known to react mainly with SH groups of cysteine, and also with ϵ-NH_2 groups of lysine. The histone studied has been fraction F3 from calf thymus. In some experiments, calf thymus histone fractions F1 and F2b have also been used for comparison. The complete sequence of calf thymus histone F3 shows it to contain two cysteinyl residues. The two SH groups can form intra- and intermolecular S–S bridges [14–17], a fact which has been correlated with some possible biological functions [18]. Other interesting structural features for this protein are its capability to form some α-helix (25–30% at relatively high ionic strength) and the tendency to aggregate as the salt concentration increases [19]. These characteristics indicate that calf thymus histone F3 may have a defined but modifiable conformation, and this paper corroborates such assertion for the regions where the cysteinyl residues are localized.

2. Materials and methods

Calf thymus histone fractions F1, F2b and F3 were obtained in this Laboratory using method II of Johns [20], and, when necessary, purified either by precipitation with acetone or by chromatography through CMC [21]. *N*-ethyl maleimide (Schuchart analytical grade) was used without further purification. The spin label *N*(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide was purchased from Synvar Associates, Palo Alto, California.

The histone fractions were labeled as follows: solid spin label (0.8 mg) was added to 20 mg of the histone fraction dissolved in 20 ml of 24 mM phosphate buffer, pH 6.8. After stirring for 1 hr at room temp. and 6 hr at 5°, the solution was dialyzed exhaustively

* Presented at the Gordon Research Conferences 1972 on "Nuclear Proteins, Chromatin Structure and Gene Regulation".

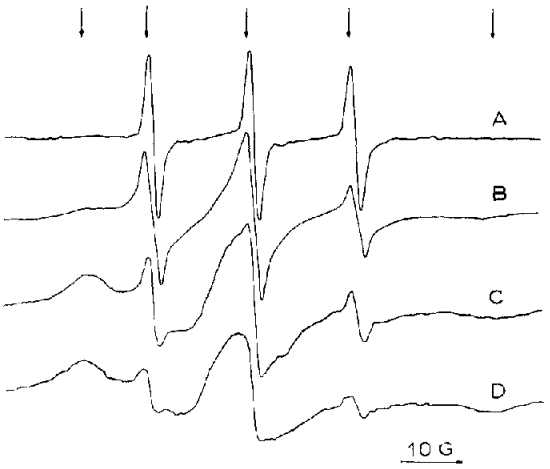

Fig. 1. EPR spectra of spin labeled histones: A) F1 in H_2O, pH 6.8; B) F3 in H_2O, pH 6.8; C) F3 in 0.024 M phosphate buffer, pH 6.8; D) F3 in 1 M NaCl containing 0.024 phosphate buffer, pH 6.8.

against the required final solvent in the cold several times, in order to remove the unreacted spin label.

The calculated maximum yield of labeling corresponds to a molecular ratio of spin label to protein of 1.6 for F2b and F3 and of 3.0 for F1.

The reaction of F3 with *N*-ethyl maleimide (NEM) was accomplished by adding 0.4 mg of NEM to 8 mg of F3 dissolved in 8 ml of 24 mM phosphate buffer, pH 6.8 and stirring 30 min at room temp. and 9 hr at 5°. After standing overnight, the solution was dialyzed exhaustively in the cold, and then reacted with the spin label as described above.

Electron paramagnetic resonance (EPR) measurements were carried out on a Varian E-4 spectrometer

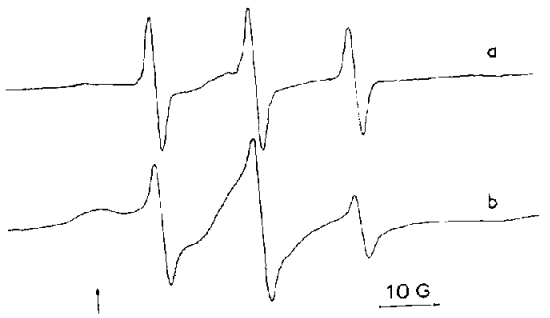

Fig. 2. EPR spectra of spin labeled histones: a) F3 previously treated with NEM in 0.0024 M phosphate buffer, pH 6.8; b) F3 in 0.0024 M phosphate buffer, pH 6.8.

(Instituto de Química Orgánica, Patronato Juan de la Cierva, CSIC, Barcelona), operating at 9.442 GHz, at room temp.

3. Results and discussion

Fig. 1 shows the EPR spectra of F1 in H_2O pH 6.8, and of F3 in H_2O pH 6.8, in 0.024 M phosphate buffer pH 6.8, and in 1 M NaCl containing 0.024 M phosphate buffer pH 6.8. The hyperfine multiplets found for F1 (fig. 1A) correspond to a free spin label in a medium of low viscosity. Similar behaviour was found for different ionic strengths up to 1 M NaCl. F2b gave the same results as F1. These findings indicate that the spin label reacting groups for these two lysine-rich histones are external ones and, consequently the paramagnetic resonance of the attached spin label is not affected by any protein environment. Thus no tertiary structure of F1 and of F2b can be visualized by the method used here.

On the other hand the EPR spectra for F3 at low ionic strength (figs. 1B and 1C) show a combination of the spectra of the free spin label in a medium of high viscosity and in a medium of complete immobilization. As can be seen, there are two broad signals (indicating highly restricted motion) flanking the sharper hyperfine lines. These sharp hyperfine components show variable intensities of the derivative signals (indicating incomplete motional restriction).

On increasing the salt molarity up to 1 M NaCl (fig. 1D), the spectrum of the spin labeled F3 shows a gradual predominance of the two broad outermost lines. This indicates that at high ionic strength the spin labels attached to the protein molecule are in a completely restricted motion.

Decreasing the pH from 6.8 to 3, or increasing the urea concentration up to 6 M, reduces the two broad outermost signals but not the asymmetry of the three sharper hyperfine lines. This indicates that at low pH or at high urea concentration the attached spin labels have a partial motional freedom.

In order to demonstrate that the cysteinyl residues are preferentially spin labeled, histone F3 was previously treated with *N*-ethyl maleimide and then labeled with the free radical. The EPR spectrum of the final solution is shown in fig. 2. The number and shape of the derivative signals are in this case similar

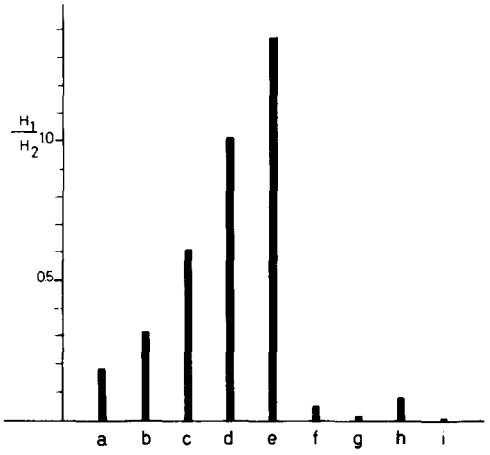

Fig. 3. Relationship between intensities of the derivative signals of the EPR spectra of spin labeled histones: a) F3 in H_2O, pH 6.8; b) F3 in 0.0024 M phosphate buffer, pH 6.8; c) F3 in 0.024 M phosphate buffer, pH 6.8; d) F3 in 0.25 M NaCl containing 0.024 M phosphate buffer pH 6.8; e) F3 in 1.0 M NaCl containing 0.024 M phosphate buffer, pH 6.8; f) F3 previously treated with NEM in 0.0024 M phosphate buffer, pH 6.8; g) F3 in 6 M urea; h) F1 in H_2O, pH 6.8; i) F2b in H_2O, pH 6.8

to those shown for histones F1 and F2b, thus indicating that, when SH groups are blocked, there is some labeling of lysyl residues in calf thymus histone F3. The spin labels do not show the effect of restriction of motion, indicating that they are external in the molecule.

Fig. 3 shows a summary of the results in terms of the ratio of intensities of the first and second EPR derivative lines for the different experiments. This ratio is a measure of completely immobilized relative to partially immobilized spin label.

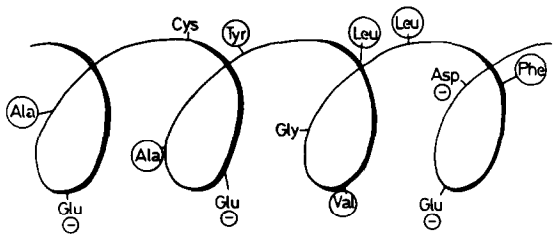

Fig. 4. Spatial distribution of residues 94–106 of histone F3 in an α-helix.

The results show that cysteinyl residues are buried in two different environments, one being a shallow depression on the surface of the protein molecule and the other being a deep crevice. At least two conformational models for the histone F3 molecule can be given, which are consistent with the presence of two kinds of crevices containing cysteine. One possibility is the presence within the population of molecules of two stable conformations, each one having a different type of crevice. A second possibility, and perhaps the more plausible, is the existence of both types of crevices in every molecule. By increasing the ionic strength one cysteinyl residue could be buried either by a conformational change of the protein or by histone self-aggregation. On the other hand a low pH or a high urea concentration would cause a partial opening up of the molecule and an increase in mobility.

Since the primary structure of F3 is known [3], suggestions may be made about the localization of a deep crevice. The cysteinyl residues are separated by 13 residues in a nonbasic region of 29 residues, but preferentially Cys-96 is present in a region of a high potential for helix formation (residues 94–106). Fig. 4 schematically shows how these residues are distributed along an α-helix. Provided that this helix is naturally occurring, it is evident that it may act as a promoting centre for tertiary structure. In fact, all the residues lying on the same side as Cys-96 are hydrophobic and therefore capable of hydrophobic interactions. On the other hand, two pairs of acidic amino acids, separated by Val-101 are lying on the other side of the helix, and may well give electrostatic interactions with two of the eight pairs of basic residues present in the whole molecule. It is tentatively concluded, therefore, that Cys-96 is situated in a stabilized deep crevice, whereas Cys-110 may be present in a more opened region of the molecule.

Frequently, the active sites containing cysteine of spin labeled enzymes and other globular proteins have an EPR behaviour similar to that described for histone F3 [22]. Therefore, although it is not suggested any enzymatic function for histone F3, it becomes apparent the relevance of the present findings towards a better understanding of the biological function of this histone within the chromosomal complex.

Acknowledgements

The authors wish to thank Dr. E.M. Bradbury for helpful discussion of the manuscript, and Dr. M. Ballester for facilities in using the EPR equipment.

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