Native State of High Mobility Group Chromosomal Proteins 1 and 2 Is Rapidly Lost by Oxidation of Sulfhydryl Groups during Storage†

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ABSTRACT: Oxidized forms of non-histone chromosomal proteins high mobility group 1 (HMG1) and HMG2 were detected by high-pressure liquid chromatography of preparations stored at 4 °C for 1 day. The oxidized form of each was found to have two free sulfhydryl groups, while the freshly prepared native form of each contained four. The native, reduced state could be maintained during storage by the addition of ethylenediaminetetraacetic acid or reducing agents.

High mobility group 1 (HMG1)[1] and HMG2 are abundant non-histone chromosomal proteins. The amino acid sequences of the two proteins are about 80% homologous, one of their interesting features being a continuous sequence of 35–40 aspartic acid and glutamic acid residues (Walker et al., 1980; Walker, 1982). Although their function is unknown, some studies have implicated HMG1 and -2 in the regulation of transcription (Vidali et al., 1977; Kleinschmidt et al., 1983). In addition, HMG1 and -2 levels have been reported to change in vivo early in the process of differentiation (Seyedin & Kistler, 1979; Seyedin et al., 1981).

On the basis of their abundance, it has been postulated that HMG1 and -2 proteins may have a structural role in chromatin (Walker et al., 1980). The proposed structural function lends importance to studies of the conformations of HMG1 and -2 [see Bradbury (1982) for a review] and to their interactions with other chromatin components. Examples of the latter are preferential binding to single-stranded DNA (Boone et al., 1982; Javaherian et al., 1979; Hamada & Bustin, 1985; Isackson et al., 1981) and selective complex formation with H1 histones (Shooter et al., 1974; Smerdon & Isenberg, 1976; Yu & Spring, 1977; Cary et al., 1979).

Obviously, it is important in studies of macromolecular interactions to work with HMG1 and -2 proteins in their native state. Since no functional assay is available, physical criteria must be used to monitor their denaturation. As reported here, two of the four sulfhydryl groups present in both HMG1 and HMG2 proteins are unusually sensitive to oxidation under common laboratory conditions. The oxidation markedly changed the elution behavior of HMG1 and -2 proteins on RP-HPLC and therefore probably would affect the macromolecular interactions of these proteins as well.

Experimental Procedures

Isolation of HMG1 and -2. The HMG1 and -2 protein preparation used for chromatography was obtained from steer thymus by the second method of Nicolas and Goodwin (1982), which employs 0.74 M perchloric acid extraction followed by acetone precipitation between 78% and 86%. The acetone precipitate was dissolved in and dialyzed against distilled water, lyophilized, and stored at −20 °C.

HPLC. Most HPLC experiments were performed on a Beckman Model 100A dual-pump chromatography system equipped with a Hitachi 100-40 spectrophotometer and an Altex C-RIA integrator. The eluent was monitored by the absorbance at 210–215 nm. A Brownlee RP300 C_8 reversed-phase column (4.6 × 220 mm, 10-μm beaded silica, 300-Å pore size) was used for most separations. Essentially identical elution profiles were obtained with a Vydac C_4 300-Å column.

Proteins were injected in 10 mM Tris, pH 7.2, with or without DTT or EDTA, and eluted at room temperature with a 60-min linear gradient from 25% to 45% acetonitrile in water [both containing 0.2% (v/v) trifluoroacetic acid] at a flow rate of 1 mL/min.

Titration of Sulfhydryl Groups. Free sulfhydryl groups were measured by the method of Ellman (1959), monitoring at 412 nm the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with the sulfhydryl groups.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was done according to the method of Laemmli (1970) with 12.5% acrylamide–0.8% bis(acrylamide) gels.

Protein Concentration. Protein concentration was determined by amino acid analysis on a Beckman 121 analyzer after hydrolysis of the proteins in 6 N HCl, 110 °C, 20 h.

Results

A mixture of HMG1 and -2 proteins, isolated from steer thymus and stored in Tris buffer that contained 1 mM EDTA, was resolved on a C_8 RP-HPLC column. Under these conditions, the two major components present eluted at 28 and 32 min; several minor components were also present (Figure 1). When EDTA was omitted from the storage buffer, a second pair of prominent peaks appeared in the chromatogram at 24 and 30 min (Figure 2A). As the EDTA-free HMG solution was allowed to stand for longer periods of time, these new peaks became increasingly prominent (Figure 2B) while the major peaks originally observed decreased, eventually to levels below the detection limit of our analyses (Figure 2C). Samples stored for several weeks at 4 °C showed no further change in chromatographic behavior.

When DTT was added to a stored sample that had shown major peaks only at 24 and 30 min, the components that eluted at 28 and 32 min were regenerated (Figure 3). After 5 days

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[1] Abbreviations: HMG, high mobility group; HPLC, high-pressure liquid chromatography; RP, reversed phase; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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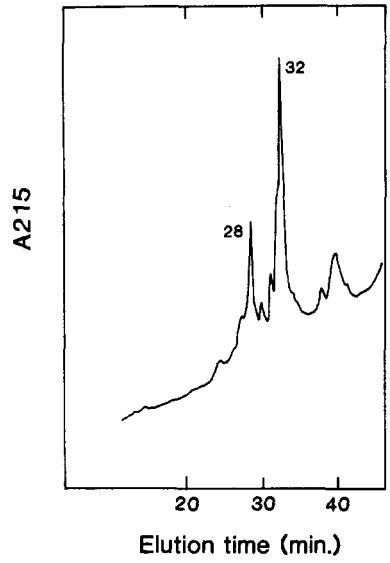

FIGURE 1: RP-HPLC separation of HMG1 and -2 proteins. About 20 μg of a preparation of HMG1 and -2 proteins in Tris buffer containing 1 mM EDTA was injected onto the RP-HPLC column. Major peaks are labeled according to their elution times.

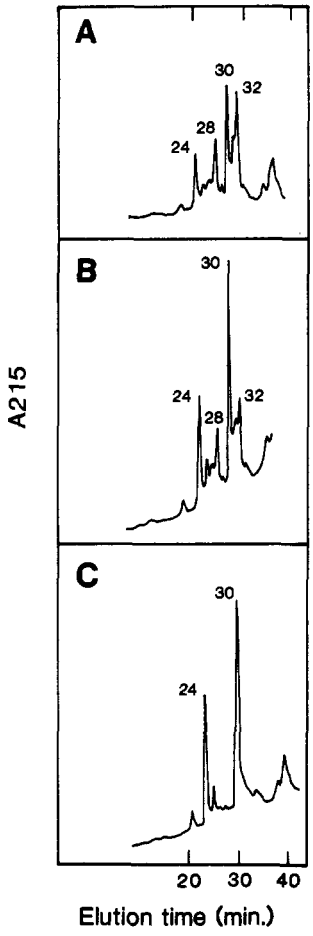

FIGURE 2: Effect of the storage of HMG1 and -2 solutions in the absence of EDTA. About 20 μg of HMG1 and -2 proteins was chromatographed after storage (4 °C) in EDTA-free buffers for (A) 2 h, (B) 5 h, or (C) 2 days. Major peaks are labeled with elution times.

in EDTA-free Tris buffer, an HMG mixture gave the chromatogram shown in Figure 3A. DTT was then added to 1 mM. After 2 days in the presence of DTT, the mixture again contained major components that eluted at 28 and 32 min (Figure 3B). Although storage in 1 mM EDTA prevented the

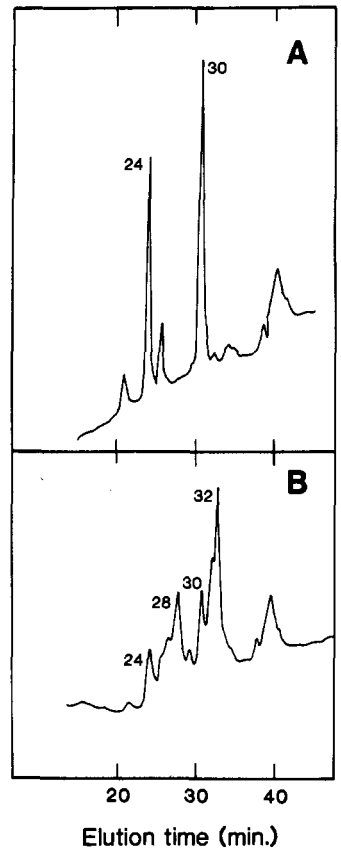

FIGURE 3: Effect of DTT on HMG1 and -2 chromatography. (A) Chromatography of 20 μg of HMG1 and -2 proteins after 5 days at 4 °C in the absence of EDTA. (B) Same as in (A) but stored an additional 2 days after addition of 1 mM dithiothreitol.

transformation of HMG1 and -2 for up to about 4 weeks, the addition of 15 mM EDTA instead of DTT could not cause restoration of peaks at 28 and 32 min even after 3 weeks at 4 °C. We therefore postulated that the observed chromatographic shift was due to an oxidation of HMG1 and -2 proteins catalyzed by metal ions rather than a conformational change promoted directly by the binding of divalent cations.

To determine which major components present in fresh extracts gave rise to which of the major components present in partially oxidized HMG preparations, the individual components were isolated by RP-HPLC, and their chromatographic behavior was assessed separately after further exposure to oxidizing conditions (Figure 4). The four major forms of HMG1 and -2 proteins in a partially oxidized extract were separated by RP-HPLC (Figure 4A) and individually rechromatographed immediately (Figure 4B, profiles 1 and 3; Figure 4C, profiles 1 and 3). Each rechromatographed component was lyophilized, redissolved in 10 mM Tris, pH 7.2, and stored at 4 °C to allow oxidation to occur. Figure 4B, profiles 2 and 4, and Figure 4C, profiles 2 and 4, show the effects of oxidation on the chromatographic behavior of four components that had originally eluted at 24, 28, 30, and 32 min. The chromatographic behavior of the components which eluted at 24 min (Figure 4B, profile 1) and 30 min (Figure 4C, profile 1) remained unchanged after storage under conditions that allowed oxidation (Figure 4B, profiles 1 and 2; Figure 4C, profiles 1 and 2). These components, therefore, are considered to be the products of the oxidation reaction. The component which originally eluted at 28 min (Figure 4B, profile 3) gave rise under oxidizing conditions to one that eluted at 24 min (Figure 4B, profile 4). We therefore conclude that the components eluted at 24 and 28 min represent the same

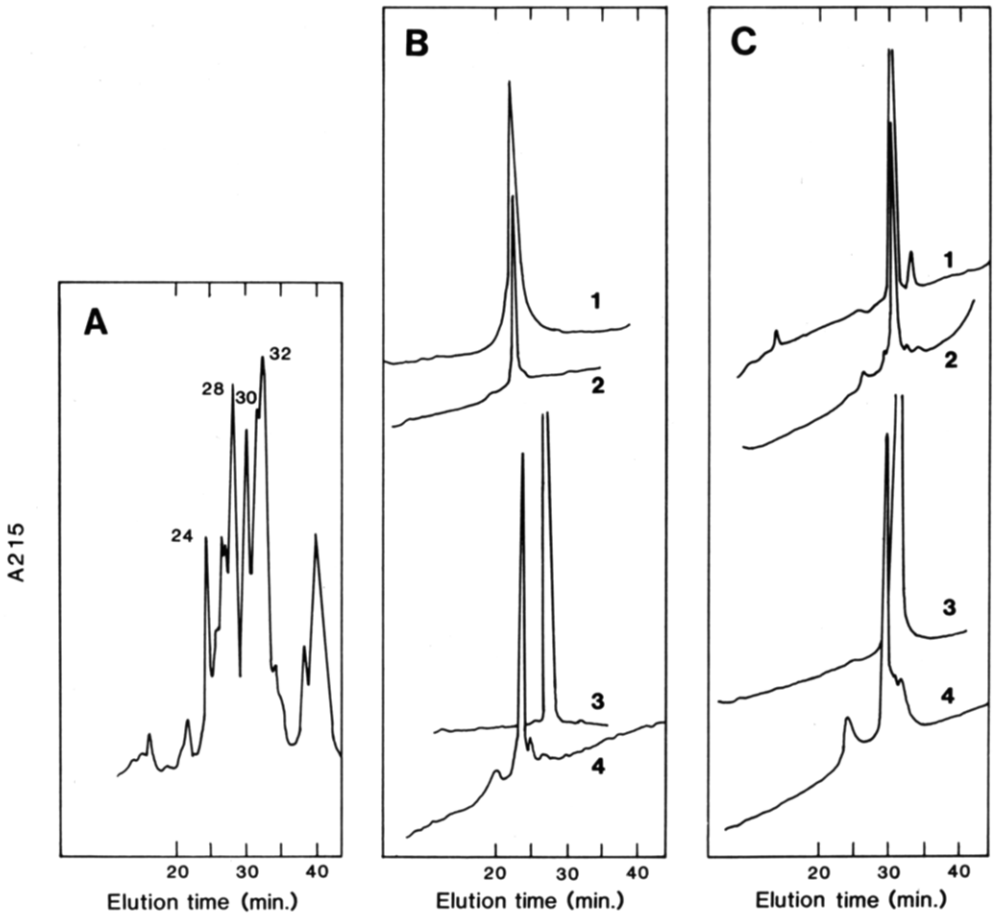

FIGURE 4: Identification of peaks in profiles of oxidized and reduced HMG1 and -2 proteins. Peak fractions (elution times labeled) were collected from the chromatogram (A) of a partially oxidized sample (250 μg). Each fraction was rechromatographed immediately, and the peak fractions were collected again. The samples were then left at 4 °C for several days in the absence of EDTA or DTT and rechromatographed again. (B) Rechromatography of the 24-min peak immediately (profile 1) and after incubation (profile 2); rechromatography of the 28-min peak immediately (profile 3) and after incubation (profile 4). (C) Rechromatography of the 30-min peak immediately (profile 1) and after incubation (profile 2); rechromatography of the 32-min peak immediately (profile 3) and after incubation (profile 4).

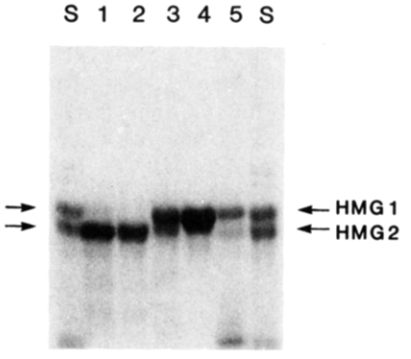

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of components isolated by RP-HPLC (Figure 3A) from an oxidized sample of HMG1 and -2 proteins. Lanes marked S, starting material. Lanes 1, 2, 3, 4, and 5 contain, respectively, peaks at 20, 24, 25, 30, and 34 min.

protein in oxidized and reduced form, respectively, and that components eluted at 30 and 32 min (Figure 4C, profiles 3 and 4) form a similar oxidized/reduced pair.

To identify the proteins represented by the peaks in the chromatogram, oxidized HMG1 and -2 proteins were separated by RP-HPLC, and each peak was collected and analyzed electrophoretically (Figure 5). The component that eluted at 24 min had a mobility on $NaDodSO_4$ gels which matched that of HMG2 protein; the component which eluted at 30 min corresponded to HMG1 protein. Minor components were also analyzed. Proteins that matched HMG1 electrophoretically

Table I: Sulfhydryl Groups per Molecule of HMG Protein[a]

	reduced	oxidized
HMG1	4.4	2.4
HMG2	3.6	2.3

[a] Results for HMG1 are an average of three titrations; results for HMG2 are an average of two.

were found in chromatographic fractions which eluted at 25 and 34 min, while others that matched HMG2 occurred in fractions eluted at 20, 25, and 34 min. A number of small peptides also eluted at 34 min. The broad peak at 40 min was composed of a large number of small peptides and a small amount of histone H1. Microheterogeneity in HMG1 and -2 proteins has been reported previously (Elton & Reeves, 1985a; Mayes, 1982).

The oxidation that caused the change in elution pattern of HMG1 and -2 proteins involved cysteine residues. The number of sulfhydryl groups per molecule of protein was measured for both the oxidized and reduced HMG1 and HMG2 proteins by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Both HMG1 and -2 proteins contained four sulfhydryl groups per molecule in the reduced form and two sulfhydryl groups in the oxidized form (Table I). Clearly, the transformation observed by RP-HPLC represents the formation of a disulfide bridge in each protein. The similarity in electrophoretic mobility of each major component in the presence or absence of 2-mercaptoethanol before and after oxidation (data not shown) rules out the formation of substantial levels of inter-

molecular disulfide bridges. The disulfide bridge formed during storage must, therefore, be intramolecular.

Discussion

We have found that HMG1 and -2 proteins both contain four sulfhydryl groups, two of which are easily oxidized under common laboratory conditions. The ease of oxidation, for example, 24 h at pH 7, 4 °C, may explain why other workers have estimated that these proteins each contain only two half-cystine residues (Walker et al., 1980; Walker, 1982). The evidence for two half-cystine residues was derived primarily from titration of sulfhydryl groups by *p*-(chloromercuri)-benzoate (Walker et al., 1976). Selective cleavage of each protein by 2-nitro-5-thiocyanatobenzoic acid produced five fragments (Walker et al., 1976). This number of fragments might be taken to represent the three fragments expected from complete cleavage at two half-cystine residues plus two fragments from partial cleavage. It is more straightforward to interpret them as the five fragments expected from complete cleavage at four half-cystine residues. Only the latter interpretation is consistent with our finding of four sulfhydryl groups in freshly prepared samples of HMG1 and -2 proteins. Apparently, the HMG1 and -2 samples titrated by Walker et al. (1976) showed only two sulfhydryl groups because two others were lost to oxidation during preparation or storage. Elton and Reeves (1985a) developed an HPLC purification method for HMG1 and -2 proteins very similar to the one we employed for the detection of sulfhydryl oxidation of these proteins. In subsequent work (Elton & Reeves, 1985b), these authors demonstrated that addition of DTT to purified HMG1 and -2 proteins changed their mobilities on acid–urea gels and that these proteins eluted differently from an RP-HPLC column in the presence or absence of DTT. The oxidation detected by Elton and Reeves undoubtedly represents disulfide formation in HMG1 and -2 proteins.

The ease with which two of the sulfhydryl groups are oxidized in fresh preparations of HMG1 and -2 proteins, combined with the fact that they are reduced in the native (freshly isolated) state, is a strong indication that the nucleus maintains a relatively reducing environment. Proteins of the nuclear matrix (Kaufmann & Shaper, 1984) and histone H3 (Palau & Dabán, 1974) have been shown to be present in the nucleus in their reduced forms, and they too undergo oxidation under mild conditions.

Because the nucleus maintains a reducing environment, it is unlikely that disulfide bonds in HMG1 and -2 proteins are of physiological significance. Nevertheless, the rapid oxidation of HMG1 and -2 proteins is of considerable importance to in vitro studies of the conformation and function of these proteins. The marked effect of oxidation on chromatographic behavior suggests that there might be profound differences between the oxidized and reduced forms of HMG1 and -2 with respect to their interactions with other macromolecules. Those differences might underlie disparate reports on properties of HMG proteins such as complex formation with H1 histone (Shooter et al., 1974; Smerdon & Isenberg, 1976; Yu & Spring, 1977; Cary et al., 1979) or the relative binding affinities of HMG1 and -2 for single-stranded DNA (Boone et al., 1982; Javaherian et al., 1978, 1979; Isackson et al., 1981; Butler et al., 1985). Investigators should guard against the rapid loss of the native state by the use of chelating or reducing agents.

Registry No. EDTA, 60-00-4; DTT, 3483-12-3.

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