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Communication

Conformational stability of six truncated cHMG1a proteins studied in their mixture by H/D exchange and electrospray ionization mass spectrometry★✪

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The high mobility group (HMG) proteins are abundant non-histone components of eukaryotic chromatin. The presence of C-terminal acidic tails is a common feature of the majority of HMG proteins. Although the biological significance of the acidic domains is not clear, they are conferring conformational and metabolic stability to the proteins *in vitro*. Moreover, the length and net charge of the acidic tails affect the strength of HMG protein interaction with DNA. Synthesis of an insect HMG protein by standard recombinant technology in bacteria leads to a mixture of the intact protein (cHMG1a-(1–113) (I)) and a series of its degradation products truncated at the C tail: cHMG1a-(1–111) (II); cHMG1a-(1–110) (III); cHMG1a-(1–109) (IV); cHMG1a-(1–108) (V); cHMG1a-(1–107) (VI); cHMG1a-(1–106) (VII). The proteins differ from each other only by the number of amino-acid residues at the C-terminal tail. We used H/D exchange mass spectrometry to characterize the stability of the proteins directly in their mixture. The results show that the proteins I–V and VII have very similar conformations. The protein VI is less compact and exchanges its protons faster than the others. It may be concluded that the C-terminal tail influences the conformation of the cHMG1a protein and that individual residues in this part of the protein play a key role in its compactness.

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Abbreviations: ESI, electrospray ion-mass spectrometry; HMG, high mobility group.

Electrospray mass spectrometry (ESI-MS) is a basic tool for studying biomolecules such as proteins, peptides and other non-volatile compounds. In contrast to other methods (NMR, FT-IR) ESI-MS enables simultaneous analysis of more than one substance in solution (Szewczuk *et al.*, 2001). This may be very practical in the case of a mixture of homologs which are difficult to separate (for example proteins from a biological source). Therefore, ESI-MS is a method of choice in checking the purity of proteins derived from biological material. In recent years ESI-MS is often used as a tool to study three-dimensional structure by monitoring hydrogen/deuterium (H/D) isotope exchange (Wang & Tang, 1996). Such experiments provide information about protein stability, dynamics and protein folding (Wang & Tang, 1996; Miranker *et al.*, 1993).

A protein in water solution continuously exchanges some of its hydrogens with water. Hydrogens in the protein can be divided into three groups. The first group consists of those hydrogens covalently bonded to carbon atoms that are not exchangeable. The second group comprises hydrogens in side chains, exchanging with water so fast that they cannot be followed easily. Amide hydrogens in the protein backbone peptide bonds belong to the third group. The exchange rate of hydrogen is closely related to its local environment. The exchange half-lives of these hydrogens vary from fractions of a second to years, which is in the range for convenient determination by mass spectrometry. Proteins with open structures have amide hydrogens that are more accessible to the solvent (D_2O), and their exchange proceeds at higher rates. Native tightly folded proteins have fewer accessible amide hydrogens, leading to a lower uptake of deuterium (Niemirowsky *et al.*, 1999). As the backbone amide hydrogens cover the whole protein evenly, they can provide conformational information for all its parts. Since the molecular mass of a molecule increases by 1 Da on each deuteration, mass spectrometry is an important tool for monitoring H/D exchange reactions.

In the present work we attempted to examine differences in conformation among several cHMG1a proteins in their mixture. The high mobility group (HMG) proteins are the most abundant non-histone components of chromatin. The members of this group are thought to have various functions related to modulation of transcription, DNA integration, and recombination. Since these proteins have an ability to induce strong bends and unwind DNA, they are called architectural components of chromatin. Previously, we found that phosphorylation of C-terminal tails influences biochemical and physical properties of the proteins as well as their conformational stability (Wiśniewski *et al.*, 1999; Schwanbeck *et al.*, 2001). Synthesis of the insect cHMG1a by standard recombinant technology (producing eukaryotic proteins in the *Escherichia coli* bacteria) leads to a mixture of the intact protein (cHMG1a-(1–113) (**I**)) and a series of its six truncated analogues (Table 1). The proteins differ from each other only by the number of amino-acid residues at the C-terminal tail. We used H/D exchange mass spectrometry to characterize conformational differences among the proteins directly in their mixture.

EXPERIMENTAL

Overexpression in bacteria and purification of cHMG1a. The coding region of the cHMG1a protein was cloned into the expression vector pET3a and the protein was overexpressed in BL21(DE3) cells as described by Wiśniewski & Schulze (1994). The C-terminal deletions resulted from the processing of the protein by an undefined exo-proteinase(s) in the bacterial cells (Wiśniewski & Schultze, 1994).

The proteins were isolated by extraction with 5% $HClO_4$ in three freezing-thawing cycles from bacteria expressing cHMG1a. The

Table1. The sequences, masses, and number of exchangeable protons of cHMG1a protein and its six truncated analogues

Symbol	Protein	Molecular mass (Da)		Sequence concluded from the observed molecular mass	Number of exchangeable protons
		calculated	observed		
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
I	cHMG1a (M)	12915	12915	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD**EEEDESD**	233
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
II	(M – **SD**)	12712	12713	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD**EEEDE**	229
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
III	(M –**ESD**)	12583	12584	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD**EEED**	227
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
IV	(M -**DESD**)	12468	12468	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD**EEE**	225
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
V	(M –**EDESD**)	12339	12340	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD**EE**	223
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
VI	(M –**EEDESD**)	12210	12210	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD**E**	221
				AEKPKRPLSAYMLWLNSARESIKKENPDFKVTEIAKKGGELWRGMKDKSEWEAKAAKM	
VII	(M –**EEEDESD**)	12081	12082	KEEYEKAMKEFERNGGDKSSGASTKKRGKAAEKKKPAKKSKKKDSEDD	219

cell supernatants were treated with HCl to its final concentration 0.35 M, and proteins were precipitated with 6 volumes of acetone and dried. Crude extracts were separated on a cation-exchange column (Poros SP20, 4.6 × 100 mm, PerSeptive Biosystems) using a 0.3–1 M NaCl gradient in 25 mM sodium borate, pH 9.4. After a 30-min incubation at 20°C the samples were concentrated and desalted on Sep-Pack Plus C18 cartridges (Waters) using 70% (v/v) CH_3CN, 0.1% (v/v) CF_3COOH/H_2O as the eluent. Finally, the proteins were chromatographed on a reverse-phase C_{18} Zorbax SB-300 column using a linear CH_3CN gradient in 0.1% (v/v) CF_3COOH/H_2O as described (Wiśniewski & Schultze, 1994; Wiśniewski *et al.*, 1999; Schwanbeck *et al.*, 2001).

H/D exchange. The deuterated solvents deuterium oxide (99.9% D) and acetic acid (98% D) used in this study were obtained from Sigma and Aldrich. The H/D exchange experiments were carried out in a manner similar to that described by Niemirowsky *et al.* (1999). A stock solution of the seven proteins mixture at 11 mg/ml in 10 mM $HCOONH_4$ buffer (pH 6.3) was prepared. The exchange experiments were carried out at 4°C. Deuteration was started by a 10-fold dilution with 10 mM $HCOOND_4$ in D_2O. The final concentration of D_2O was 90%. After a certain period of time (0.5, 1.35, 2.23, 8.13, 15.22, 60, 120, 240 min., 8 h) 15 μl solutions were probed. The exchange reaction was arrested by acidification to pD = 3 by 5% CH_3COOD in D_2O (Niemirowsky *et al.*, 1999; Resing & Ahn, 1988) and the sample was introduced into an electrospray mass spectrometer.

Mass spectra. Mass spectra were recorded on a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source. The ion spray needle was maintained at 4.5 kV. Nitrogen gas was used to evaporate the solvent from the charged droplets. The resulting spectra were transformed using a BioWorks software package (Finnigan).

RESULTS AND DISCUSSION

MS of the mixture of truncated cHMG1a proteins

The expression in *Escherichia coli* of the cHMG1a protein from the native *Chironomus tentans* sequence resulted in the synthesis of

the entire protein (residues 1–113; (**I**)) and incomplete proteins in which the acidic tail was truncated. Similar results observed for HMG1 proteins (Bianchi, 1991; Payet & Travers, 1997) suggested that these proteins were sensitive to proteolysis (Bianchi, 1991). The spectrum of our material confirmed the presence of a mixture of these proteins (see Fig. 1A). In the deconvoluted spectrum (Fig. 1B) there are

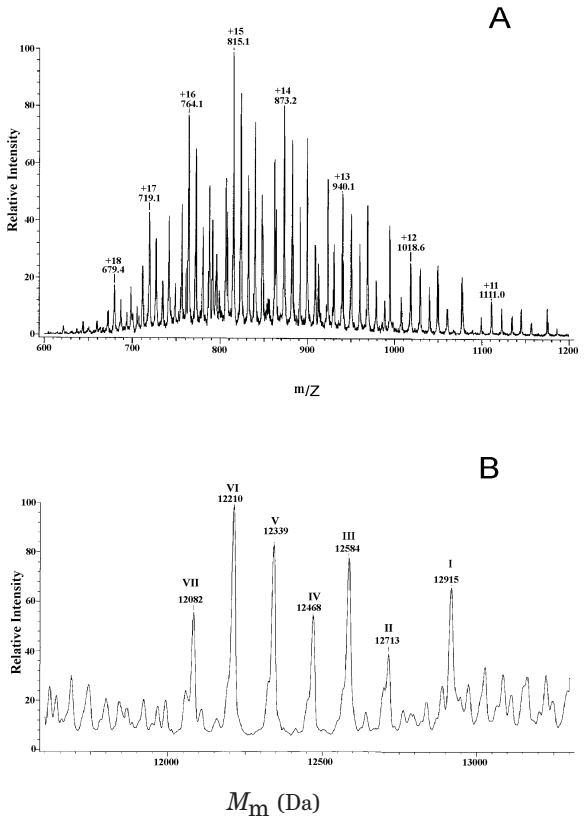

Figure 1. A. ESI-MS spectrum of the protein mixture investigated.

The marked peaks correspond to the protein **VI**, which is the most abundant.

B. Deconvoluted spectrum of A showing the presence of seven components.

The signals correspond to the cHMG1a protein (**I**) and its six analogs (**II–VII**) truncated at C terminus.

seven peaks corresponding to masses of cHMG1a and its truncated forms. An analysis of the ESI-MS spectrum shows that the most abundant peek corresponds to protein **VI**. The calculated molecular masses are presented in Table 1.

H/D exchange

The cHMG1a protein (**I**) consists of 113 amino-acids residues and has 233 exchangeable protons. The sequences of the other proteins studied and the numbers of their exchangeable protons are presented in Table 1. The effect of shortening the C-terminal tail of cHMG1a was analyzed by the H/D exchange at 4°C monitored by ESI-MS. The difference in the molecular mass of the deuterated and undeuterated protein provides the average number of exchangeable protons that did exchange with deuters. In our experiments the final concentration of D_2O was 90%. It has previously been shown (Ramanathen *et al.*, 1997) that D_2O concentration does not affect the exchange extent in proteins. The number of unexchanged hydrogens can be therefore calculated by using the following simple equation:

$$H(r) = (M_a - M_o)/0.9 + E_p$$

where: M_a — average molecular mass of the protein, M_o — observed molecular mass of the exchanged protein, E_p — number of exchangeable protons (data in Fig. 2).

We initiate hydrogen exchange of the proteins by 10-fold diluting the non-deuterated solvent with its deuterated analogue. In our approach the proteins were already folded in solution prior to the addition of the deuterated solvent. This approach should reduce the possibility of conformational refolding that may occur in the case of direct dissolving of lyophilized material in D_2O.

In this study hydrogen exchange was assayed by electrospray mass spectrometry on a mixture of cHMG1a and its truncated analogs. No further isolation of the truncated forms was necessary since the peaks were well separated (Fig. 1). Hydrogen exchange study on a mixture of proteins may be very useful for a comprehensive study of compounds with different molecular mass. Differences in exchange rates of such compounds are not sensi-

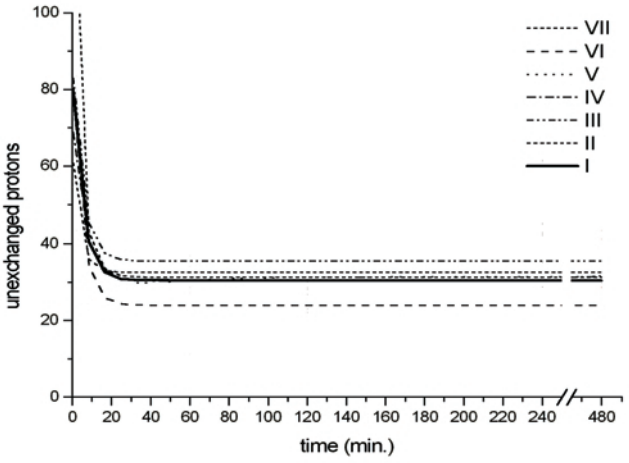

Figure 2. H/D exchange curves of proteins: I–VII.

The data were approximated by fitting to the equation with two exponential terms:

$$H(r)=H_1\exp(-k_1t)+H_2\exp(-k_2t)+H_3$$

(where: H_1 – protons deuterated with fast kinetic constant k_1, H_2 – protons deuterated with a lower kinetic constant k_2, H_3 – protons fully protected from deuteration and back-exchanged hydrogen atoms).

tive to conditions of the experiment (like deuterium back-exchange, pH, deuterium concentration, temperature, spectrometer calibration drifting, etc.), since these conditions should affect all the components to the same extent. On the other hand, study of complex mixtures may be impractical if peaks overlap . It may happen in the case of small differences in molecular masses of individual components in the mixture, salt contamination, and compounds with multimodal kinetics of hydrogen exchange. The above does not concern the protein mixture studied, since the molecular masses of its components differ significantly, samples were well desalted and all the peaks were sharp enough, even on the relatively low-resolution quadrupole mass analyzer used.

Electrospray mass analysis of truncated analogs **II–V** after deuterium exchange showed that the degree of deuterium incorporation is relatively low and similar to that of the intact protein cHMG1a (**I**). This indicates no detectable structural changes upon removal of the first five residues from the protein C-terminus. Thus, conformations of compounds **I–V** are partially rigid and many exchangeable hydrogens are not accessible to the deuterated solvent. In contrast, protein **VI** with the C-terminal hexapeptide **EEDESD** removed shows a higher rate of H/D exchange. This suggests that the conformation of this particular protein is less compact and approximately additional ten of exchangeable hydrogens are accessible to the deuterated solvent. An unexpected observation is that the stability of protein **VII** is similar to those of **I–V**. Therefore, the number of the labile hydrogens protected is significantly increased after removing the C-terminal Glu^{107} residue from protein **VI**.

Our results indicate that residues Asp^{106} and Glu^{107} have a significant influence on the stability of the molecule. It may be concluded that Glu^{107} is critical for the rigidity of cHMG1a, since the lack of this residue enables deuteration of a higher number of exchangeable protons (probably located inside the protein). The different extents of deuteration may suggest that Glu^{107} causes destabilization of the conformation whereas Asp^{106} positively influences the stability of the truncated protein. Our results demonstrate that the C-terminal tail of HMGA proteins may affect their 3-dimensional structure. The plots in Fig. 2 suggest that there are at least 25 protons in each protein not exchanged even after eight hours of deuteration. This should not be interpreted as number of unexchanged protons buried inside protein **VI**. The value may be affected by back-exchange of deuters caused by trace amount of vapors in the atmosphere. The back-exchange reaction is a major problem in mass spectrometric exchange studies. Resing & Ahn (1988) reported that even 33–50% deuters may back

exchange during the experiment. Although in our experiment the ionization chamber of the mass spectrometer was blown vigorously with dry nitrogen to eliminate the back exchange reaction, still the process could occur. However, back-exchange should affect the same number of deuteriums in all the proteins.

The interactions of vertebrate HMG proteins with DNA has been extensively studied. It has been found that deletion of the C-terminal stretch of mainly acidic residues 103–113 of cHMG1a resulted in an increase of fluorescence intensity of cHMG1a/102 and affecting its DNA binding (Wiśniewski & Schultze, 1994). Therefore, the highly charged C-terminal region appears to influence conformation of cHMG1a and interactions of the whole molecule with DNA.

Usually, each synthesis of a biopolymer produces, beside the desired compound, some structurally similar by-products. These by-products are eliminated during arduous purifications although they may provide many interesting data concerning the structure – stability relationship. Our results demonstrate the extraordinary powers of H/D exchange–ESI-MS experiment as a method to study crude products. One simple experiment on a complex mixture of the desired compound and its by-products may provide important information about the differences in conformation of all of the compounds present. Application of such a method to screen for proteins with desired thermodynamical properties appears to be a powerful tool in biotechnology.

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