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Oxidation of a critical methionine modulates DNA binding of the *Drosophila melanogaster* high mobility group protein, HMG-D

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Abstract HMG-D is a major high mobility group chromosomal protein present during early embryogenesis in *Drosophila melanogaster*. During overexpression and purification of HMG-D from *E. coli*, a key DNA binding residue, methionine 13, undergoes oxidation to methionine sulfoxide. Oxidation of this critical residue decreases the affinity of HMG-D for DNA by three-fold, altering the structure of the HMG-D-DNA complex without affecting the structure of the free protein. This work shows that minor modification of DNA intercalating residues may be used to fine tune the DNA binding affinity of HMG domain proteins.

Key words: High mobility group; Protein-DNA complex; Methionine oxidation; Chromosomal protein; Protein purification

1. Introduction

Methionine oxidation is a post-translation modification frequently associated with a change in activity in many biological systems [1]. Proteins including the ribosomal protein L12 [2], *E. coli* derived human stem cell factor [3], and hen egg white lysozyme [4], all lose biological activity when this modification is present. In contrast, the presence of oxidized methionine in completely functional proteins such as recombinant tissue-type plasminogen activator, recombinant interferon γ [5] and biosynthetic human growth hormone [6], indicates that this modification does not necessarily coincide with a loss of activity. Here we show that the DNA binding affinity of a high mobility group protein from *Drosophila melanogaster*, HMG-D, is attenuated by oxidation of a key methionine residue that is thought to partially intercalate DNA.

HMG-D is an abundant chromosomal protein found during the early stages of *Drosophila* embryogenesis [7,8] that uses an HMG-box DNA binding domain (Fig. 1) to bind DNA non-sequence specifically [7,9,10]. The HMG-box is found in a large sub-family of non-sequence-specific chromosomal proteins, including HMG-D (reviewed in [11]). It is also the DNA binding domain of sequence-specific transcription factors such as Lymphoid Enhancer Factor (LEF-1) [12], testis determining factor (SRY) [13], and Upstream Binding Factor (UBF) [14] (reviewed in [15]). The NMR structures of LEF-1 and SRY bound to DNA show a key intercalation event by a methionine and isoleucine, respectively [16–18]. Comparable structures of the chromosomal HMG proteins bound to DNA have been elusive [10,19].

Previous structural studies using NMR methods had been performed with a recombinant 100 amino acid form of HMG-D complexed with a 16 base pair duplex DNA fragment (GCTTATTGAAAAATCG) [10]. On addition of DNA to the protein, the NOE crosspeaks associated with the indole protons of each of the three tryptophans present in free HMG-D doubled and tryptophan 43 resonances shifted downfield (Fig. 2A and [10]). HSQC spectra of the complex revealed further resonance doubling (data not shown). These experiments indicated that two bound forms of HMG-D appeared to be in slow exchange on the NMR time scale, suggesting that HMG-D recognizes this DNA using two different binding modes. Previous biochemical studies of the interactions between HMG-D and DNA were consistent with these results. For long DNA fragments (>21 base pairs), ladders of bands were observed in electrophoretic mobility shift assays (EMSA) with HMG-D. Because multiple HMG-D molecules bind to the DNA, approximately one protein for every ten to fifteen base pairs, it was expected that HMG-D might adopt slightly different sequence-dependent modes of binding [10]. For short DNA fragments (<21 base pairs), the EMSA data used to obtain HMG-D-DNA binding constants could not be fitted with parameters indicative of formation of a simple 1:1 complex, despite the observation of only one bound complex in the gel (data not shown) [10,20,21]. Together these experiments suggested that HMG-D may bind to DNA using multiple binding modes and that complex equilibria are associated with DNA binding. Both the NMR and binding results were also consistent with the known function of chromosomal proteins which is to bind DNA at many different sites [22,23].

Here we show that HMG-D is oxidized during bacterial expression and subsequent purification of the protein, and that this oxidation decreases the DNA binding affinity of HMG-D and affects the structure of the HMG-D-DNA complex. To establish the identity of the oxidized residue, methionine 13, the separated native and oxidized forms of the protein were analyzed using mass spectrometry of proteolytic digests of the isolated proteins. By analogy with SRY and LEF-1 [16–18], Met^{13} is a crucial residue in the interaction of HMG-D with DNA. Using quantitative EMSA, we show that oxidization of Met^{13} decreases the DNA binding affinity of the protein by a factor of three. We further demonstrate by NMR and quantitative EMSA, that the unoxidized HMG-D binds to a short duplex DNA fragment using a single binding mode and that a one-to-one complex is formed between

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HMG-D and a short disulfide crosslinked DNA fragment [20].

2. Materials and methods

2.1. Protein expression and purification

The gene sequence encoding the first 74 and 100 amino acid residues of HMG-D was sub-cloned into the T7 expression system vector pET13a as described previously [24,25]. HMG-D-74 and HMG-D-100 were overexpressed in *E. coli* strain BL-21(DE3) (Novagen) in LB media at 37°C. Protein purification, with dithiothreitol present in all buffers, proceeded as outlined previously [25,10] except for the addition of an HPLC step to separate the oxidized and unoxidized protein. After the DEAE (Pharmacia) column, the protein was pooled, dialyzed against deionized water, and concentrated in a Centricon 3 (Amicon) microconcentrator. The native and oxidized forms of the protein were separated by RP-HPLC (LKB) using a Waters μBondapak C18 column. The protein was eluted isocratically at 3.5 ml/min with buffer containing 31% acetonitrile and 69% 0.2 M dibasic sodium phosphate, pH 2.3. Fractions corresponding to the two forms of the protein were separately pooled, loaded onto the SP-Sepharose column, and eluted as before. Purified protein was pooled, dialyzed, and concentrated.

2.2. Mass spectrometry

Samples of the intact protein were analyzed by Electrospray (ES) mass spectrometry using a Micromass Quattro triple quadrupole mass spectrometer equipped with a Megaflow electrospray ion source. Protein samples were diluted in water and acetonitrile containing 0.1% v/v formic acid. The multichannel analyzer mode was used to acquire mass spectra between m/e 500–2000 with a scan time of 10 s. Resolution corresponded to a peak width at half height of 1.4 Da for m/e 893. The mass spectra were transformed to a molecular mass scale using the Micromass MaxEnt Electrospray software. Samples of the proteolyzed protein were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry on a Micromass TofSpec time of flight instrument. The matrix was 4-Hydroxy-α-cyanocinnamic acid saturated in acetonitrile and water (66/33) with 0.1% trifluoroacetic acid. 1 μl of a 2 pm/μl solution of the digest in 0.1% trifluoroacetic acid was mixed with 1 μl of the matrix on the target and allowed to dry.

2.3. Proteolysis

Endoproteinase Glu-C (V8 Protease) from Boehringer Mannheim was diluted to 0.5 U/μl with water. Digestion of the oxidized and native form of the 74 construct of HMG-D took place in a 50 μl reaction volume containing 200 pmol of protein and 1.1×10^{-3} U enzyme in a 50 mM ammonium bicarbonate buffer, pH 7.8. The reaction proceeded for 18.5 h at 37°C. The samples were dried by vacuum and submitted for MALDI mass spectroscopy analysis.

2.4. NMR sample preparation and spectroscopy

The single stranded oligonucleotide components of TG16B (GCTTATTGAAAAATCG) were synthesized, purified and quantitated as reported previously [10]. The strands were annealed in a 1:1 molar ratio in 100 mM NaCl by heating the reaction mixture to 90°C for 5 min followed by slow cooling to room temperature. The duplex DNA was desalted by exchanging against water in a Microcon 3 concentrator (Amicon). NMR samples, with a molar ratio DNA: protein of 1.0:0.85, contained 1.14 mM duplex DNA and 0.97 mM HMG-D-100 in 50 mM deuterated sodium acetate, pH 5.0, 0.02% NaN_3, and 10% D_2O (v/v). NMR data were collected using a Varian VXR500S spectrometer equipped with a 5 mm dual channel inverse detection probe at 20°C. ^{1}H-NOESY spectra were recorded with the last 90° pulse replaced with a 1–1 pulse [26]. All spectra were acquired in phase sensitive mode as described previously [25,10]. Data were processed and analyzed using the FELIX1.1 software package (Biosym Technologies). ^{1}H Chemical shifts are given relative to internal sodium 2,2,3,3-[$^2H_4$]-3-trimethylsilylpropionate.

2.5. Electrophoretic mobility shift assays

Band shift assays were conducted using a 20 bp disulfide crosslinked DNA fragment kindly provided to us by Drs. Scot Wolfe and Greg Verdine [20]. The DNA was 5′ end-labeled using γ-^{32}P-ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase. Protein dilutions were made from a 10 mM stock of HMG-D in 50 mM HEPES, 0.1 M KCl, 1 mM EDTA, 100 μg/ml BSA, and 50% glycerol. 10 μl reaction mixtures contained less than 1 nM radiolabelled DNA, 2 μg/ml BSA, 5 mM sodium acetate buffer, pH 5.0. HMG-D (diluted as above) was added to give final concentrations from 0 to 200 nM. Reactions were incubated with the DNA at 22°C for 30 min. The samples were electrophoresed on a pre-run 6% polyacrylamide gel at 150 V for 45 min, dried, and then exposed to film. In order to determine the fraction of DNA bound, the gels were exposed to a phosphorimaging screen between 10 and 24 h and subsequently scanned using the Molecular Dynamics Phosphorimager and analyzed using the ImageQuant program. The intensity integrals for each band were manipulated using the Excel (Microsoft) and Kaleidagraph (Abelbeck Software) programs where the fraction of bound and free DNA was calculated and plotted for each protein concentration. The averaged data were fitted using non-linear least squares refinement methods implemented in Kaleidagraph with the single site binding equation ($Y = K^n[P_f]^n/(1+K^n[P_f]^n)$), where the equilibrium association constant is K, n is the Hill cooperativity coefficient, and the free protein concentration is $[P_f]$. The dissociation constant, K_d, was then calculated.

3. Results

3.1. Observation and separation of oxidized HMG-D species

High resolution electrospray mass spectrometry provided an explanation for the results from previous HMG-D DNA binding and NMR experiments [10]. HMG-D, overexpressed in *E. coli* is pure according to standard criteria: visualization of a single band by SDS polyacrylamide gel electrophoresis and a single peak from high resolution ion exchange chromatography (SP-Sepharose and Mono-S). However, it revealed the presence of a protein species not previously observed by other methods (Fig. 3A). The major peak at 10888.6 ± 1.4 mass units in this figure corresponds to the molecular weight of the 100 amino acid construct of HMG-D lacking the initiator methionine. A second major peak (designated with a * in panel A) at 10904.5 ± 1.3, 15.9 mass units higher than the first, suggested the presence of an oxidized form of HMG-D.

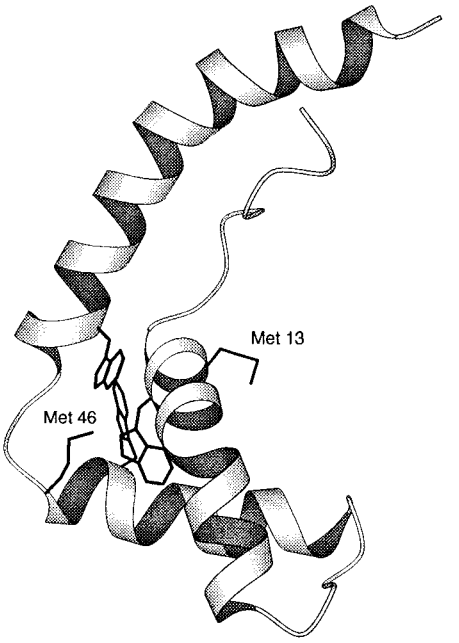

Fig. 1. Diagram of the HMG-box domain of HMG-D. The diagram was prepared from the PDB coordinates of HMG-D, 1HMA, using MOLSCRIPT [41].

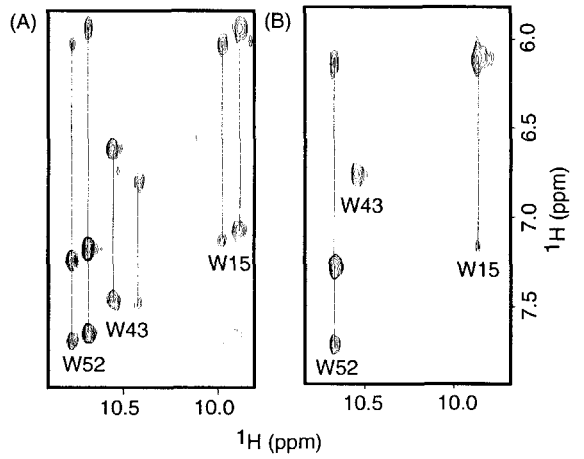

Fig. 2. ^{1}H-NOESY spectrum recorded in H_2O showing connectivities between the indole and aromatic protons of the three tryptophan residues of HMG-D. The spectra show A: HMG-D-100 bound to the DNA duplex GCTTATTGAAAAATCG and B: HPLC purified unoxidized HMG-D-100 bound to the same DNA. Due to the sample concentration and the lower signal to noise ratio obtained with the spectrometer and probe used for this particular experiment, one of the W43 NOESY crosspeaks is visible only at a lower contour level than shown in panel B.

Since HMG-D does not contain either cysteine or histidine, amino acids commonly oxidized in biological systems, one of the two remaining methionines was being oxidized to methionine sulfoxide. The relative size of the peaks in Fig. 3A was similar to the relative intensity of the two sets of NMR signals observed previously, suggesting that the oxidized HMG-D was responsible for the second set of tryptophan resonances.

HMG-D is a stable, well folded protein that can withstand denaturing purification methods. This property permits separation of the oxidized from the native species under harsh conditions without inactivating the protein. The oxidized methionine in HMG-D decreases the hydrophobicity of the protein. This hydrophobicity difference was exploited using reversed-phase HPLC chromatography where polar molecules elute prior to their more hydrophobic counterparts. Isocratic elution of the protein yielded baseline separation of two prominent protein species (data not shown). Fig. 3B shows the electrospray mass spectrometry results for the second peak in the HPLC elution profile. The peak at 10888.0 ± 1.9 Da in this figure corresponds to the native protein. Mass spectrometry analysis of the first peak in the HPLC elution profile, shown in Fig. 3C, revealed a species with an average mass 16 mass units higher than that for native HMG-D, corresponding to the oxidized form of the protein. This order of elution from RP-HPLC is expected because the oxidized protein is less hydrophobic and would elute prior to the more hydrophobic native HMG-D. Using this purification protocol, virtually all of the oxidized protein can be separated from the native protein, providing homogeneous HMG-D for further structural and thermodynamic studies.

The appearance of two distinct sets of resonances in the NMR spectrum of the HMG-D-100-DNA complex (Fig. 2A) is explained by the presence of two protein species, and suggests that the two forms of the protein produce slightly different complexes with DNA. To confirm this conclusion, we repeated NMR experiments with the HPLC purified native HMG-D-100 in complex with DNA. A single set of resonances was observed for each of the indole protons in the tryptophan indole NH region of the spectrum (Fig. 2B), corresponding to one of the two sets of peaks observed previously (Fig. 2A and [10]). Since both the oxidized and unoxidized forms of the protein bind to DNA under the conditions of the NMR experiment, the previously observed resonance doubling reflects the presence of different electronic environments within each complex. Different environments could be caused by either local electronic effects near the methionine or by more global structural differences induced by DNA binding to the oxidized and unoxidized proteins. Since other doubled resonances (data not shown) and the affected tryptophans, shown in Fig. 2A, are 10 to 12 Å away from the methionine (see Fig. 1) the chemical shift differences are unlikely to be caused solely by electronic effects, and are most likely due to slight differences in the overall structure of the complexes formed by the oxidized and unoxidized proteins. This conclu-

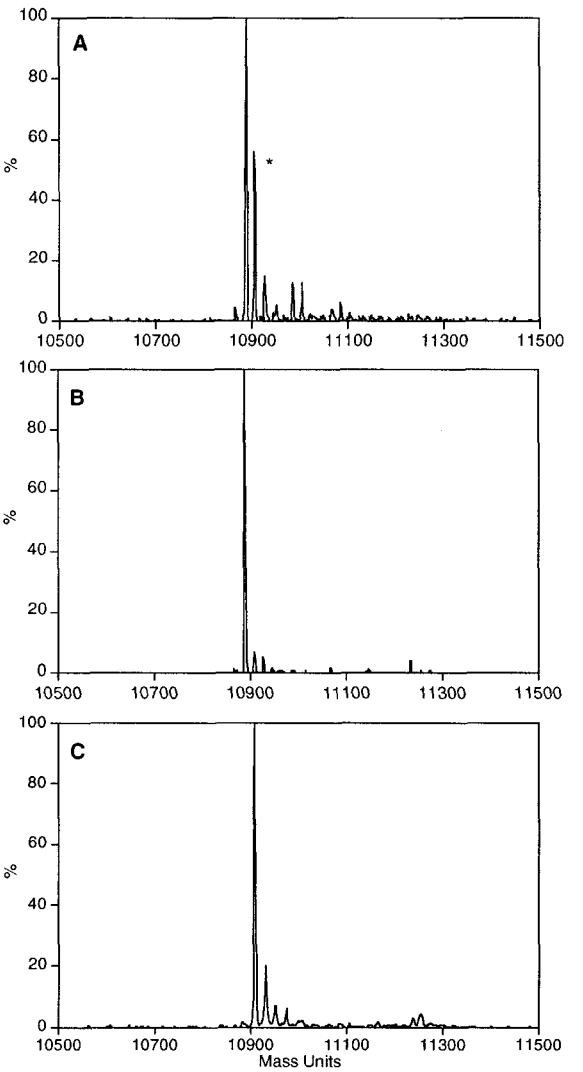

Fig. 3. Electrospray mass spectroscopy analysis of HMG-D-100. A: HMG-D-100 prior to HPLC purification. The largest peak corresponds to the native protein at 10888.6 ± 1.4 mass units. The peak designated by the * corresponds to the oxidized form of the protein at 10904.5 ± 1.3 mass units. B: Native protein after HPLC purification. C: Oxidized protein after HPLC purification.

Table 1
Peptides obtained from proteolysis of oxidized and unoxidized HMG-D-74

Fragment	Residue #	Theoretical mass	Peak 1	Peak 2
1[a]	2–21	2362.7	2380.3[c]	2363.9
2	22–26	631.7	–	–
3	27–34	857	–	–
4	35–41	715.8	–	–
5[b]	42–51	1263.5	–	–
6	52–53	333.3	–	–
7	54–69	1779	1780.2	1780.7
8	70–71	294.3	–	–
9	72–74	242	–	–
Partial digests				
1+2[a]	2–26	2976.4	–	–
1+2+3[a]	2–34	3815.4	–	–
2+3	22–34	1470.7	1471.7	1471.7
2+3+4	22–41	2168.5	–	–
3+4	27–41	1554.8	–	–
3+4+5[b]	27–51	2800.3	–	–
4+5[b]	35–51	1961.3	–	–
4+5+6[b]	35–53	2276.6	2278.8	2278.4
5+6	42–53	1578.8	1579.5	1580.2
5+6+7	42–69	3339.8	–	–
6+7	52–69	2094.3	–	–
6+7+8	52–71	2370.6	–	–
7+8	54–71	2055.3	–	–
7+8+9	54–74	2279.3	2278.8	2278.4
8+9	70–74	518.3	–	–

–, not observed.
[a]Contains Met^{13}.
[b]Contains Met^{46}.
[c]Oxidized fragment.

sion is further supported by the fact that no resonance doubling was observed during the structure determination of the free protein [10]. Finally, the single set of resonances observed in Fig. 2B, clearly demonstrates that the homogeneous native (unoxidized) HMG-D has one primary mode of binding for this duplex DNA fragment.

3.2. *Identification of the oxidized methionine and its effect on DNA binding*

The single oxidation event revealed by mass spectrometry and the observation of only two bound species in the NMR experiment suggested that only one of the two methionines, Met^{13} or Met^{46}, of HMG-D is consistently oxidized. In order to identify the oxidized residue, we performed proteolytic digestion of the isolated native and oxidized proteins with Endoproteinase Glu-C under conditions where it cleaves preferentially at the carboxylic side of glutamic acid residues. The cleavage products of the two species of HMG-D were then analyzed by MALDI mass spectrometry; these results are summarized in Table 1. Complete digestion of the protein would theoretically yield nine individual peptide fragments. Our protein was only partially digested, but all cleavage products can be explained. Fragment one is easily identified by its unique mass of 2364. It contains Met^{13} and is comprised of the residues SDKPKRPLSAYMLWLNSARE. The oxidized species yields the same fragment with a mass of 2380, 16 mass units higher than the corresponding fragment in the native protein. These results clearly indicate that Met^{13}, a surface residue on the concave face of the protein (Fig. 1), has been oxidized.

The other methionine, Met^{46}, is an integral component of the primary hydrophobic core of the protein (Fig. 1). Oxidation of this residue would be expected to lead to significant changes in the structure of the free and the bound protein. However, no resonance doubling and no indication of any heterogeneity in the hydrophobic core associated with Met^{46} was observed during the structure determination of free HMG-D-74 [25]. To confirm that Met^{46} is not being oxidized, we examined theoretical fragment 5, which contains Met^{46}. Because of an incomplete enzymatic digest, two cleavage products contain fragment 5; one is composed of the theoretical fragments 4 to 6 and the other contains fragments 5 to 6. The theoretical mass of these peptides is 2277 and 1579, respectively. Peptides with these masses were found in the cleavage products of both the native and oxidized form of HMG-D, indicating that Met^{46} is not oxidized. These results unequivocally establish that Met^{13} is the sole residue being oxidized in the HMG-D sample we obtain from standard bacterial overexpression and purification.

Oxidation of Met^{13} affects the structure of HMG-D bound to DNA (Fig. 1). By analogy with the function of this key residue in other HMG-box proteins, LEF-1 and SRY, oxidation would also be expected to hinder DNA intercalation and alter DNA binding affinity [16,27,28,17,18]. To test this hypothesis we determined the affinity of the oxidized and unoxidized HMG-D-100 for DNA using EMSA. The binding assays were performed using a 'pre-bent' 20 nucleotide disulfide crosslinked DNA fragment that had previously been used to show that HMG-D could be recruited to a single site as assayed by DNaseI footprinting methods [20]. Only one complex is visible in the band shift gel on addition of the protein (Fig. 4A) and the binding curves fit well to a one-to-one binding isotherm for each case (Fig. 4B). The dissociation equilibrium binding constant of the native and oxidized species of HMG-D-100 for DNA is 1.4×10^{-9} M and 4.3×10^{-9} M respectively with Hill coefficients of 1 indicating one-to-one

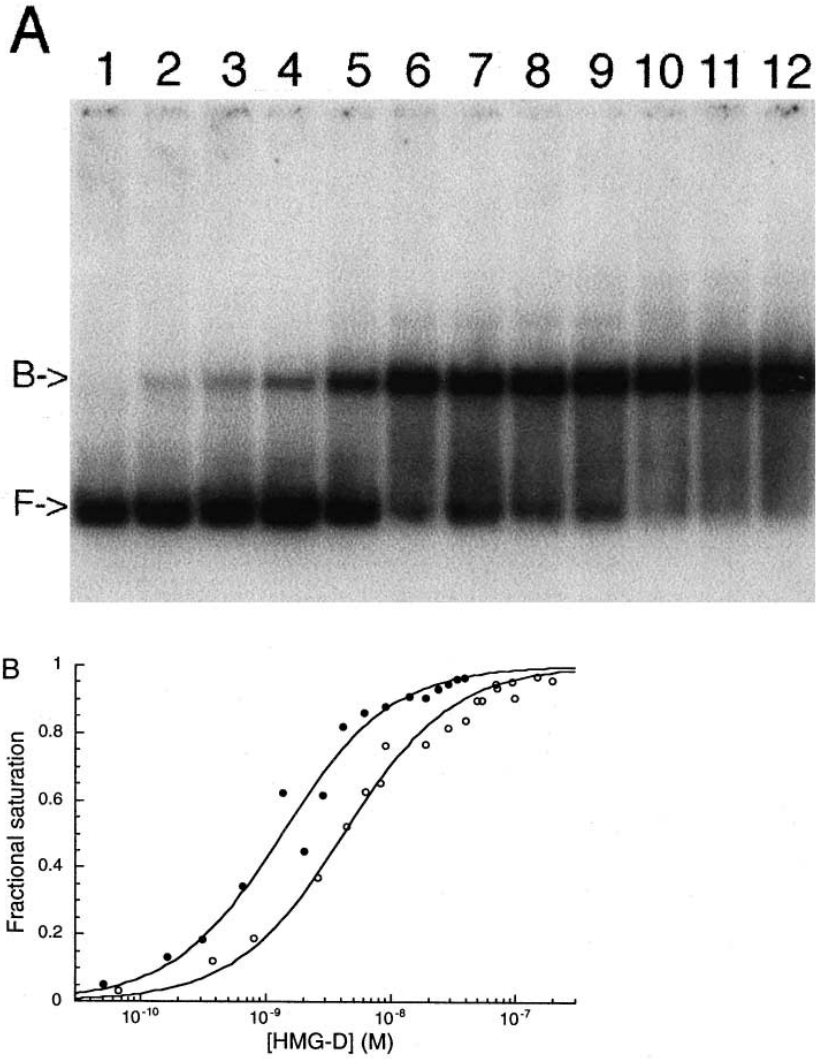

Fig. 4. Effect of oxidation on the binding affinity of HMG-D-100 for a 20 bp disulfide crosslinked DNA fragment [20]. A: Digitized image of a typical EMSA gel. Free DNA is shown in lane 1; lanes 2–12 also contain HMG-D-100 at 0.1, 0.3, 0.5, 1.0, 2.5, 3.5, 5.0, 10.0, 15.0, 25.0, 35.0 nM. Free and bound DNA are denoted by F-> and B-> respectively. B: Binding curves obtained from data similar to A for the native (six experiments) and oxidized (five experiments) species of HMG-D-100. The data were averaged and plotted as a function of free protein concentration. HMG-D-100* refers to HMG-D-100 with oxidized Met^{13}.

binding. Oxidation of Met^{13} modulates the binding affinity of HMG-D only by a factor of three.

4. Discussion

4.1. The effect of oxidation on HMG-D Met^{13} intercalation

Identification of Met^{13} as the oxidized residue in HMG-D explains why multiple binding modes of the protein are observed; whereas the putative DNA intercalation function of Met^{13} explains why the affinity of the protein is altered by such a minor modification. Sequence alignment of HMG-box proteins reveals that Met^{13} is equivalent to the isoleucine of SRY [9] and to the methionine of LEF-1 that undergo minor groove partial intercalation [16–18]. If Met^{13} does perform a comparable partial intercalation function in HMG-D, then the additional oxygen atom would be expected to alter DNA binding as observed.

The effect of oxidation of Met^{13} on DNA affinity of HMG-D is small, as expected, because the additional oxygen perturbs the structure or hydrophobicity of the methionine residue very little. Inspection of the sequences of HMG-box proteins, with the exception of UBF, reveals that several aliphatic amino acids and phenylalanine are consistently found in a position comparable to Met^{13} [9]. Even in non-HMG-box proteins, such as the purine repressor, PurR, and the TATA binding protein, TBP, leucine and phenylalanine act as minor groove intercalating residues [30–32]. Mutational studies on PurR show that replacing the critical intercalating leucine with lysine, serine, tryptophan, threonine, or arginine results in decreased repression of the purF-lacZ gene, whereas mutation to a methionine has little effect [33]. In contrast, the sex reversal mutation of SRY [27], I68T, where the equivalent to HMG-D Met^{13} has been replaced by threonine, decreases DNA binding by nearly two orders of magnitude [28]. Therefore, hydrophobic intercalating residues are important for DNA binding by HMG-box and other proteins [16,33,28,17,34]. The behavior of the conservative substitution of L > M in PurR is comparable to the change in affinity observed between the two forms of methionine in HMG-D. These studies suggest that amino acid substitution at the in-

tercalating position may be used to fine tune DNA binding affinity and shape of the DNA-protein complex.

4.2. Why is Met^{13} preferentially oxidized?

Oxidized methionines present at protein/protein or protein/ligand interfaces may not affect the structure of the free protein, but they can interfere with the interaction of the protein with its target molecules. In order to locate and assess the importance of such residues, several oxidizing agents have been used as effective tools to oxidize proteins. Hydrogen peroxide has been used to measure the kinetics of oxidation in a number of proteins [35,3]. Amiconi, using a second reagent, chloramine T, showed that oxidation of a key methionine at the $\alpha_1\beta_1$ interface of hemoglobin completely destabilizes the T state [36]. Treatment of proteins with progressively higher concentrations of a third reagent, N-chlorosuccinamide can distinguish between surface, partially exposed, and buried methionine residues [37]. Finally, Keck has shown that t-butyl hydroperoxide is a highly selective oxidant, affecting only surface exposed methionines [5]. Selective oxidation can provide insight into the position of methionine residues in a protein and their effects on biological activity.

In the case of HMG-D, Met^{13} is consistently oxidized whereas Met^{46} is not. Therefore, Met^{13} must either be intrinsically more susceptible to oxidation or less easily reduced by *E. coli* methionine sulfoxide reductases than Met^{46} [38,39]. Clearly, chemical oxidants can distinguish between surface methionines and their buried counterparts. Met^{46} resides in a well packed hydrophobic core while Met^{13} is in an exposed helix (Fig. 1). The surface area of the sulfur atom in Met^{13} is more than 25 times more exposed to a 1.4 Å probe than the sulfur of Met^{46} as determined using the exposed surface area algorithm implemented in the program ACCESS (Handschumacher and Richards, 1983). Thus, Met^{13} is a much more likely candidate for oxidation. While the position of Met^{13} in the concave region of the L-shaped protein leaves it susceptible to small oxidizing agents, it may also restrict the entry of enzymes such as *E. coli* methionine sulfoxide reductases which serve to reverse the oxidation process [38,39].

4.3. Implications for the use of recombinant proteins in biophysical studies

While methionine oxidation can be induced, it is primarily a non-enzymatic, reversible process which occurs naturally in the cell and is governed by biological oxidants and counteracting enzymes [38–40]. The stress associated with overexpressing gene products may enhance the possibility of generating oxidized methionines in the desired protein, which may lead to a change in its structure or activity. The DNA binding properties of HMG1 have been altered by use of different purification protocols, but the nature of the difference between the resulting proteins was not determined [29]. Clearly, oxidation occurs during preparation of recombinant HMG-D and alters its DNA binding function. Interestingly, many commonly used methods to assess HMG-D protein purity, including the NMR structure determination of the free protein and DNA binding analyses, gave no clear indication of heterogeneity, despite the fact that nearly one third of the protein was oxidized. The only method that ultimately proved the homogeneity of the free protein was mass spectrometry. The observation, identification and removal of the oxidized protein has allowed us to show by NMR that HMG-D binds to a short (16 base pair) linear duplex DNA fragment using a single binding mode, and will now facilitate study of the structure and mechanism of non-sequence-specific DNA recognition by HMG-D. This result is consistent with an NMR study of the HMG1 boxA domain bound to DNA which shows a single set of protein resonances corresponding to bound HMG1 [19]. Although HMG-D is still capable of binding to long linear duplex DNA fragments (>21 base pairs) and synthetic Holliday junctions using multiple DNA binding modes [10], we have verified quantitatively that HMG-D-100 forms a high affinity one-to-one complex with a 'pre-bent' DNA fragment [20], and is therefore suitable for structural studies.

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