Biophysical characterizations of human mitochondrial transcription factor A and its binding to tumor suppressor p53

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ABSTRACT

Human mitochondrial transcription factor A (TFAM) is a multi-functional protein, involved in different aspects of maintaining mitochondrial genome integrity. In this report, we characterized TFAM and its interaction with tumor suppressor p53 using various biophysical methods. DNA-free TFAM is a thermally unstable protein that is in equilibrium between monomers and dimers. Self-association of TFAM is modulated by its basic C-terminal tail. The DNA-binding ability of TFAM is mainly contributed by its first HMG-box, while the second HMG-box has low-DNA-binding capability. We also obtained backbone resonance assignments from the NMR spectra of both HMG-boxes of TFAM. TFAM binds primarily to the N-terminal transactivation domain of p53, with a $K_d$ of $1.95 \pm 0.19$ nM. The C-terminal regulatory domain of p53 provides a secondary binding site for TFAM. The TFAM–p53-binding interface involves both TAD1 and TAD2 sub-domains of p53. Helices $\alpha_1$ and $\alpha_2$ of the HMG-box constitute the main p53-binding region. Since both TFAM and p53 binds preferentially to distorted DNA, the TFAM–p53 interaction is implicated in DNA damage and repair. In addition, the DNA-binding mechanism of TFAM and biological relevance of the TFAM–p53 interaction are discussed.

INTRODUCTION

Mitochondria, which possibly have evolved from an endosymbiotically incorporated organism, have their own genome. Human mitochondrial genome (mtDNA) is a circular double-stranded DNA (dsDNA) of 16,569 bp in length (www.mitomap.org). It encodes 13 proteins that are considered essential sub-units of mitochondrial respiratory chain, two rRNAs and 22 tRNAs. The rRNAs and tRNAs are recruited to construct mitochondrial translational machineries, required for synthesis of proteins encoded by mtDNA. Best known for their role in ATP production via the process of oxidative phosphorylation (OXPHOS), mitochondria are vital for an array of fundamental cellular functions including fatty acid oxidation, biosynthesis of amino acids and heme, apoptosis and signal transduction. Maintenance of the mitochondrial genome is naturally crucial for survival. Mitochondrial dysfunctions are increasingly being associated with human diseases (1) such as cancer, diabetes, Parkinson’s disease as well as cellular senescence (2).

Human mitochondrial transcription factor A (TFAM), originally cloned as a transcription factor for mtDNA, is a multi-functional protein (3). Transcription of mitochondrial genomes initiates from three promoters: the light strand promoter (LSP) and two heavy strand promoters (HSP1 and HSP2) that are found in the 1.1 kb displacement loop (D-loop) control region of mtDNA. Transcriptional activation capability of TFAM is mediated through its high affinity to these sequence-specific DNAs and its ability to recruit mitochondrial RNA polymerase (POLRMT) as well as mitochondrial transcription factor B (TFBM) (4). In addition, TFAM serves a role in packaging mtDNA into nucleoid-like structure and its abundance in mitochondria is correlated with mtDNA copy number. According to classic strand-asymmetric model (also termed strand-displacement model) of mtDNA replication (5–7), transcription is coupled to replication. TFAM is, therefore, implicated in mtDNA replication. Accumulating evidences suggest that TFAM is a DNA damage sensor since it binds more strongly to oxidatively damaged DNA and cisplatin adducts. Its interaction with tumor suppressor p53 is...
believed to play a part in DNA damage and repair (8). mtDNA is physiologically more vulnerable compared with nuclear DNA, in view of its close proximity to OXPHOS sites. It is considered that ~1–5% of mitochondrially consumed oxygen is converted to reactive oxygen species (ROS) due to electron leaks from respiratory chain. In addition, mtDNA is more prone to chemical damage since lipophilic cations tend to accumulate in mitochondrial matrix, facilitated by negative membrane potential at matrix-side. TFAM has been reported to protect mtDNA from these oxidative and chemical attacks. Moreover, TFAM displayed higher affinity to branched DNA structure (e.g. 4-way junction) and might affect DNA recombination events.

TFAM belongs to a large and diverse superfamily of high-mobility group (HMG) protein (9). Within this superfamily, three structurally distinct classes of HMG proteins have been defined: HMG–nucleosome-binding family (HMGN), HMG-AT-hook family (HMGA) and HMG-box family (HMGB). Mammalian HMG-box containing proteins can further be sub-divided into two major groups. The first group consists of HMGB-type non-sequence-specific DNA-binding proteins with two HMG-box domains and a long highly acidic C-tail (e.g. HMGB1). The second group is highly diverse and consists of mostly sequence-specific proteins having a single HMG-box and no acidic C-tails (e.g. SRY, SOX). Nevertheless, there exist proteins that have up to six copies of the HMG-box domain (e.g. UBF1). As a HMGB protein, TFAM is unique in that it binds specific sequence preferentially, but contains two HMG-box domains. The gene of TFAM spans about 10 kb and consists of seven exons and six introns (10). Exon 5 can splice alternatively, resulting in two TFAM isoforms (11).

In this article, we report biophysical characterization of TFAM and its interaction with tumor suppressor p53. In addition to examinations of TFAM’s oligomerization state and thermal stability, we performed solution-based DNA binding of TFAM using fluorescence spectroscopy and circular dichroism (CD) spectropolarimetry. For the first time, TFAM was studied using nuclear magnetic resonance (NMR). We obtained backbone resonance assignments from the NMR spectra of both HMG-box domains of TFAM. We were, therefore, able to map the p53- and DNA-binding sites on HMG-box domain of TFAM. Similarly, we located binding region of HMG-box on p53. The DNA-binding mechanism of TFAM and biological relevance of TFAM–p53 interaction are also discussed.

**MATERIALS AND METHODS**

**Nucleic acids**

The sequences of single-stranded DNAs (ssDNA) and dsDNA used in this study were summarized in Supplementary Table S1. All DNAs were HPLC-purified and were purchased from either Sigma Genosys or Eurogentec.

**Cloning and expression of HMG1, HMG2, NEx-HMG2, TFAM and TFAM-ΔC**

Gene fragments, corresponding to residues 50–118 (HMG1), 155–219 (HMG2), 144–219 (NEx-HMG2), 50–246 (TFAM) and 50–219 (TFAM-ΔC) of TFAM (NCBI NP_003192), were PCR-amplified and cloned into pET24a-HLTV vector using restriction sites BamHI and EcoRI. N-terminal protein region (residues 1–49) was expressed in E. coli (DE3). Cells were grown in M9 media supplemented with 50 μg/ml kanamycin, at 37°C. When OD600 reached 0.5–0.6, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce protein expression and temperature was lowered to 25°C. After 24 h, cells were harvested and pellets were frozen in liquid nitrogen.

**Purification of HMG1, HMG2, NEx-HMG2, TFAM and TFAM-ΔC**

HMG1, HMG2 and NEx-HMG2. Cells were thawed at 4°C in buffer A [50 mM Tris–HCl pH 7.4, 300 mM NaCl, 5 mM imidazole, 1% (v/v) 2-mercaptoethanol] supplemented with DNase, RNase and protease inhibitor (Roche), and were lysed by high-pressure homogenization (EmulsiFlex-C5, Avestin). After centrifugation, we loaded supernatant onto HiTrap HP column (GE Healthcare) pre-equilibrated with buffer A, protein a eluted with linear gradient of imidazole (0.005–1 M) in buffer A. The sample was diluted with buffer B [25 mM Tris–HCl pH 7.4, 5 mM DTT, 10% (v/v) glycerol] to adjust NaCl concentration to ~100 mM and loaded onto HiTrap Heparin HP column (GE Healthcare) pre-equilibrated with buffer B containing 100 mM NaCl. Upon washing with 5 CV of buffer B containing 100 mM NaCl, protein was eluted with linear gradient of NaCl (0–1 M) in buffer B. Fractions containing protein of interest were pooled and loaded onto HiLoad 26/60 Superdex 75 column (GE Healthcare) pre-equilibrated with buffer C [25 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM DTT, 10% (v/v) glycerol]. All chromatographic steps were performed at 4°C. Protein concentration was estimated using extinction coefficient of 16 960/M/cm. 13C,15N-labeled sample for NMR was produced in M9 media using [13]C6-D-glucose and [15]NH4Cl as carbon source and nitrogen source, respectively.

TFAM and TFAM-ΔC. Both proteins were purified as above, with the following modifications: upon TEV cleavage, TFAM-containing sample was loaded directly onto HiTrap Heparin HP column (GE Healthcare) pre-equilibrated with buffer B containing 300 mM NaCl. After washing with 5 CV of buffer B containing 300 mM NaCl, TFAM was eluted with buffer B containing 1 M NaCl. In the case of TFAM-ΔC, protein sample was diluted with
buffer B to adjust NaCl concentration to ~150 mM after TEV cleavage. TFAM-ΔC-containing sample was loaded onto HiTrap Heparin HP column (GE Healthcare) pre-equilibrated with buffer B containing 150 mM NaCl, followed by washing with 5 CV of identical buffer. TFAM-ΔC was eluted with linear gradient of NaCl (0–1 M) in buffer B. Both TFAM and TFAM-ΔC were subsequently subjected to size exclusion using HiLoad 26/60 Superdex 75 column (GE Healthcare) pre-equilibrated with buffer D [25 mM Tris–HCl pH 7.4, 300 mM NaCl, 5 mM DTT, 10% (v/v) glycerol]. Protein concentrations were estimated using extinction coefficients of 35 410/M/cm (TFAM) and 33 920/M/cm (TFAM-ΔC).

Expression and purification of N-terminal domain of p53
N-terminal domain of p53 (p53N, residues 1–93) was expressed and purified as previously reported (13).

Analytical ultracentrifugation
Equilibrium sedimentation experiments were performed on a Beckman XL-1 ultracentrifuge by using Ti-60 rotor and 6-sector cells at the following speeds: [35 000, 40 000 and 45 000 r.p.m.] (HMG1 and HMG2), [25 000, 30 000, 35 000 r.p.m.] (TFAM) and [28 000, 30 000, 34 000 r.p.m] (TFAM-ΔC). Data were collected at 5°C, following absorbance at 230 nm, 280 nm and interference. The sample volume was 110 µl and concentrations tested were 5–400 µg/ml. Buffer conditions were 25 mM Tris–HCl pH 7.4 and 150 mM NaCl. For samples containing TFAM, 1 mM DTT was supplemented. Samples were considered to be at equilibrium as judged by a comparison of several scans at each speed. Data were processed and analyzed by using UltraSpin software, which is available from our web site (www.mrc-cpe.cam.ac.uk).

CD spectropolarimetry and thermal melting
Thermal stabilities of various protein constructs (HMG1, HMG2, NEx-HMG2, TFAM and TFAM-ΔC) were studied through CD spectropolarimetry. Thermostated JASCO J-815 CD spectropolarimeter was used with constant nitrogen gas purged. Protein concentration used was 10–20 µg/ml, in buffer containing 25 mM HEPES pH 7.4 and 150 mM NaCl. For sample containing TFAM, 1 mM 1,4-dithioerythritol was supplemented. Thermal denaturation curves for all proteins were obtained by recording CD spectra at 222 nm, from 2°C to 98°C at a temperature ramp rate of 60°C/h. To study conformational change of TFAM upon DNA binding, CD spectra of TFAM were recorded in the absence or presence of 28-bp dsDNA (Supplementary Table S1). Wavelength scans were performed at 20°C, in 0.2 nm steps from 194 to 260 nm, in 25 mM sodium phosphate pH 7.4, 100 mM KCl and 1 mM 1,4-dithioerythritol. The protein concentration was 10 µM and DNA concentration was 4 µM. The spectra recorded were corrected for the spectra of the buffer and of the DNA. All CD measurements were made in quartz cuvette with 1 mm path length.

DNA- or p53N-binding of HMG1, HMG2, NEx-HMG2, TFAM and TFAM-ΔC
The binding of various protein constructs (HMG1, HMG2, NEx-HMG2, TFAM and TFAM-ΔC) to either ssDNA, dsDNA or p53N was performed by using fluorescence anisotropy. Measurements were recorded on FluoroMax-3 spectrofluorimeter (Jobin-Yvon–Horiba) equipped with a Hamilton Microlab Titrator, at 37°C (unless otherwise stated) in buffer containing 25 mM Tris–HCl pH 7.4, 150 mM NaCl and 5 mM DTT. Protein was titrated into a cuvette containing either 1.2-ml fluorescein-labeled DNA [initial concentration 100 nM (HMG1, HMG2 and NEx-HMG2) or 25 nM (TFAM and TFAM-ΔC)] or AlexaFluor 546-labeled p53N (initial concentration 50 nM), as described earlier (13). Excitation/emission wavelengths for fluorescein and AlexaFluor 546 were 480/530 and 540/569 nm, respectively. Fluorescence anisotropy was calculated from the fluorescence intensities and binding analysis was performed using either a simple 1-state binding isotherm or Hill equation (13,14).

Intrinsic fluorescence measurements
Intrinsic fluorescence measurements were performed with a Cary Eclipse fluorescence spectrophotometer (Varian). Scans were recorded using excitation wavelength of 280 nm and emission wavelength ranged from 300 to 400 nm. Slit widths for both excitation and emission were kept at 5 nm, with a photomultiplier voltage of 900 V. Concentrations of TFAM and/or DNA were 500 nM, in buffer containing 25 mM Tris–HCl pH 7.4, 150 mM NaCl and 5 mM DTT.

NMR spectroscopy
NMR spectra were acquired with a Bruker Avance-II + 700-MHz spectrometer equipped with a 5 mm cryogenic inverse probe and single-axis gradients.

NMR protein backbone assignment. Backbone 1H, 13C and 15N resonance assignments of HMG1 and HMG2 were recorded at sample temperatures of 15°C and 5°C, respectively. Protein concentration used was 200 µM in buffer containing 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM DTT and 5% (v/v) D2O. Sequence-specific backbone assignments were achieved using 2D 1H,15N-heteronuclear single quantum coherence (HSQC), 3D HNCO, 3D HN(CA)CO, 3D CBCA(CO)NH, 3D HNCACB, 3D HN(CO)CA and 3D HNCA. All spectra were processed and analyzed with TopSpin version 2 (Bruker) and Sparky package (http://www.cgl.ucsf.edu/home/sparky/), respectively. The automated assignment program MARS was used to initially assign the backbone resonances (15). Near-complete backbone resonance assignments were achieved for HMG1 (~97%) and HMG2 (~88%).

1D 1H-NMR experiment. Effective molecular weights in solution were estimated by measuring 1H T2 relaxation time constants for amide protons, using the 1D-spinecho method (16). Spectra were recorded for ~75 µM TFAM.
and TFAM-ΔC in 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM DTT and 5% (v/v) D<sub>2</sub>O, at 5°C. T<sub>2</sub> estimates were obtained by comparing peak intensities in spectra recorded with spin-echo delays of 400 μs or 2 ms.

**HSQC.** To map the HMG1-binding site on p53N, 1H,15N-HSQC spectra were recorded with protein in a buffer of 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM DTT and 5% (v/v) D<sub>2</sub>O at 5°C. Concentrations of 15N-labeled p53N + non-labeled HMG1 were 100 μM. For identifying p53N-binding site or DNA-binding site on HMG1, 1H,15N-HSQC spectra were recorded with protein in a buffer of 50 mM MES pH 6.8, 100 mM NaCl, 5 mM DTT and 5% (v/v) D<sub>2</sub>O at 5°C. In both control (13C,15N-labeled HMG1) and sample ([13C,15N-labeled HMG1 + non-labeled p53N] or [13C,15N-labeled HMG1 + non-labeled 28-bp dsDNA]) runs, concentrations of HMG1, p53N and DNA were 100 μM. All spectra were externally referenced based on the frequency of the water resonance.

**Cell lines and confocal immunofluorescence microscopy**

Human colon epithelial cells, HCT116 p53<sup>+/+</sup>, were grown and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM + GlutaMax-1, Invitrogen), supplemented with 10% FCS. Cells were sub-cultured at a ratio of 1:8 in standard 6-well plate and cells were grown on glass cover slips overnight. Media were replaced with either fresh DMEM media or DMEM supplemented with cisplatin (20/50 μM, freshly prepared in DMSO). After 24 h, cells were subjected to immunofluorescence staining as described earlier (13). Images were collected on a BioRad Radiance confocal microscope.

**Chemical shift-based homology modeling**

Chemical shift database search was performed using a chemical shift comparison algorithm SimShiftDB (17,18). SimShiftDB analyses each possible pairing of the target protein with each template in the database and retrieves a list of matching proteins, scored by a measure of statistical significance. In addition, SimShiftDB provides a pair-wise chemical shift alignment that maps a set of residues from the target protein to a set of residues from the template structure. We used the chemical shifts data for TFAM HMG1 to scan the PDB template database with SimShiftDB. The search retrieved the structures of UBF1-HMG4 (PDB 1wgf) and TOX-HMG (PDB 2co9) with highest scores of 15e–15 and 14e–14, respectively. A composite alignment derived from the alignments of the top-scoring templates was used as an input for Modeller (19,20). Twenty-five models were generated. The stereo-chemical qualities of all generated models were assessed using PROCHECK (21). The Ramachandran plot shows 98.2% of the residues in the most-favored region, 1.7% in the additional allowed region and 0.2% in the disallowed region. Pair-wise structure superimpositions were performed by using TopMatch (22). PyMOL (http://www.pymol.org/) was used for visualization of the structures.

**RESULTS**

**Domain architecture of TFAM**

TFAM contains two HMG-box domains (Figure 1A). A basic 36-residue linker connects these tandem HMG-box domains, followed by a 27-residue carboxyl-terminal tail that is also rich in basic amino acid residues. In order to examine the DNA-binding propensity and function of individual domains, we have created a series of TFAM deletion constructs (Figure 1A): ‘HMG1’ (residues 50–118, first HMG-box domain), ‘HMG2’ (155–219, second HMG-box domain), ‘NEx-HMG2’ (144–219, second HMG-box domain with a partial linker at its N-terminus), ‘TFAM’ (50–246, full-length mitochondrial form of TFAM) and ‘TFAM-ΔC’ (50–219, mitochondrial form of TFAM without C-terminal tail). N-terminal protein region (residues 1–49) was predicted to be MTS using MITOPROT [http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html; (12)] and was, therefore, removed from our protein constructs. In other words, proteolytic cleavage site upon mitochondrial transportation of TFAM was assumed to be C49P50 in the present study. Nevertheless, some research groups regard 42F–43S as the proteolytic cleavage site (23,24). Further, experimental evidences exist indicating that mitochondrial targeting determinants of TFAM may extend beyond the cleavable MTS (24). Thus, the precise presequence of TFAM remains to be clarified. All proteins used in this study were extensively purified to near homogeneity, as judged from SDS-PAGE (Figure 1B). Cell lysate was subjected to DNaseI digestion followed by SDS-PAGE and immunoblot analysis using the anti-TFAM antibody (Figure 1B).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) A schematic diagram of various TFAM deletion constructs used in this study. Dashed box indicates region deleted in the shorter TFAM isoform. (B) SDS-PAGE of various TFAM deletion constructs on a NuPAGE<sup>®</sup> Novex 4–12% Bis–Tris gel.
Table 1. Apparent molecular weights of various TFAM deletion constructs, obtained from equilibrium sedimentation AUC

|                  | Single-exponential model |                  | Double-exponential model |                  |
|------------------|--------------------------|------------------|--------------------------|------------------|
|                  | Apparent $M_w$ (kDa)     | $F^a$            | Apparent $M_w$ (kDa)     | $F^a$            |
|                  |                          | of first species |                          | of second species|
| HMG1 (Calculated $M_w = 8.63$ kDa) |                      |                  |                          |                  |
| 50.0 µM          | 8.84 ± 0.04              | 1.02             | –                        | –                |
| 200.0 µM         | 8.76 ± 0.01              | 1.02             | –                        | –                |
| HMG2 (Calculated $M_w = 8.07$ kDa) |                      |                  |                          |                  |
| 5.0 µM           | 8.68 ± 0.05              | 1.08             | –                        | –                |
| 50.0 µM          | 7.41 ± 0.03              | 0.86             | –                        | –                |
| 200.0 µM         | 8.94 ± 0.01              | 1.11             | –                        | –                |
| 400.0 µM         | –                        | –                | 7.47 ± 0.07              | 0.93             |
|                  |                          |                  | 13.52 ± 0.15             | 1.68             |
| TFAM (Calculated $M_w = 23.95$ kDa) |                      |                  |                          |                  |
| 5.0 µM           | –                        | –                | 25.29 ± 0.37             | 1.06             |
| 10.0 µM          | –                        | –                | 25.02 ± 0.75             | 1.04             |
| 50.0 µM          | –                        | –                | 24.71 ± 0.14             | 1.03             |
| 100.0 µM         | –                        | –                | 24.78 ± 0.08             | 1.03             |
| 150.0 µM         | –                        | –                | 22.88 ± 0.30             | 0.96             |
| 189.4 µM         | –                        | –                | 23.18 ± 0.11             | 0.97             |
|                  |                          |                  | 44.71 ± 0.44             | 1.87             |
| TFAM-AC (Calculated $M_w = 20.69$ kDa) |                      |                  |                          |                  |
| 10.0 µM          | 20.76 ± 0.08             | 1.00             | –                        | –                |
| 50.0 µM          | 20.91 ± 0.03             | 1.01             | –                        | –                |
| 100.0 µM         | 21.07 ± 0.03             | 1.02             | –                        | –                |

$F = \text{Apparent } M_w / \text{calculated } M_w$

*Data were fitted to either a single-exponential model or a double-exponential model.


treatment prior to three chromatographic steps (HisTrap HP column, HiTrap Heparin HP column, HiLoad 26/60 Superdex 75 column) to ensure DNA-free proteins for biophysical studies.

**DNA-free TFAM exists as monomer–dimer in equilibrium**

During the purification of HMG1, TFAM and TFAM-AC (Figure 1A), we have observed anomalous elution profiles in size exclusion chromatography (Supplementary Figure S1). HMG1 was found to have an elution volume that indicated a size of a tetramer, whereas TFAM and TFAM-AC displayed elution volumes consistent with sizes of dimers. In both HMG2 and NEx-HMG2 (Figure 1A), the elution volumes showed approximately the same elution volumes of monomer. We have also noticed that elution volume of TFAM was slightly affected by the ionic strength of buffer used (Supplementary Table S2). Increased buffer’s ionic strength resulted in reduced protein mobility, and therefore, a delayed elution from gel filtration column. HMG proteins were named on the basis of their irregular electrophoretic mobility in polyacrylamide gels. As a member of HMG proteins, the observed behavior of TFAM and its deletion constructs (particularly HMG1 and TFAM-AC) in Superdex column is probably not surprising. Nevertheless, knowing the stoichiometric state of TFAM is of paramount importance to understand how TFAM functions in promoter or mitochondrial DNA (mtDNA) binding. It has been suggested that TFAM exists as monomer from sedimentation velocity analysis (23) and glycerol gradient sedimentation analysis (25). Kaufman et al. concluded that TFAM was a dimer from size exclusion chromatography (albeit an inappropriate methodology for HMG proteins analyses) and proposed that TFAM dimerization was likely mediated through the predicted coil-coil domain that resides within the second HMG-box domain (26). These disagreements prompted us to use equilibrium sedimentation analytical ultracentrifugation (AUC) to unequivocally determine the stoichiometry of TFAM and its deletion constructs. Our results showed that individual HMG-box domain (HMG1 or HMG2) of TFAM exists as monomer in solution (Table 1). The data of both HMG1 and HMG2 were fitted well to single-exponential models, using partial specific volume ($\upsilon_B$) of 0.7319 (HMG1) or 0.7107 (HMG2) and solvent density ($\rho$) of 1.007 at 5°C (all values were calculated using Sednterp software). Averaged apparent molecular weights ($M_w$) of 8.80 and 8.34 kDa were obtained for HMG1 and HMG2, respectively. These values correspond to calculated molecular weights of HMG1 monomer ($M_w = 8.63$ kDa) and HMG2 monomer ($M_w = 8.07$ kDa). Attempt to fit the TFAM data to single-exponential model was not successful (Figure 2A). However, the data was fitted well to double-exponential model using $\upsilon_B = 0.7296$ and $\rho = 1.007$ (Figure 2B), indicating that TFAM is neither a homogeneous solution of monomer nor dimer. Based on the averaged $M_w$ found (24.31 and 47.15 kDa), TFAM exists as monomer (calculated $M_w = 23.95$ kDa) and dimer (calculated $M_w = 47.90$ kDa). Surprisingly, TFAM-AC was a monomer from our AUC experiments. Upon fitting to single-exponential model ($\upsilon_B = 0.7296$ and $\rho = 1.007$), an averaged $M_w$ of 20.91 kDa was acquired, corresponding to monomeric size of TFAM-AC (calculated $M_w = 20.69$ kDa). To revalidate these
findings, we derived an effective molecular weight of both TFAM and TFAM-ΔC from NMR $^1$H T$_2$ relaxation time constant estimates. Judging from the spectral profiles (number of peaks and peak positions), TFAM and TFAM-ΔC were structurally indistinguishable, as expected. Overlapping spectra at different temperatures clearly indicated that TFAM-ΔC, but not TFAM, was homogeneous solution (data not shown). Combining data from size-exclusion, AUC and NMR, we proposed that: (i) DNA-free TFAM exists as monomer–dimer in equilibrium; (ii) self-association of TFAM is mediated through its C-terminal tail; and (iii) high-mobility of TFAM observed in size-exclusion is contributed mainly by its first HMG-box domain.

**TFAM is thermally unstable**

We next examined the thermal stability of TFAM through circular dichroism. Since TFAM is rich in helical content from PSIPRED prediction [Supplementary Figure S2; (27)], we followed the change of ellipticity at 222 nm as temperature was raised from 2°C to 98°C. The reversibility of thermal denaturation was checked by reheating the sample (rescan), after cooling the sample to its starting temperature. Supplementary Figure S3 illustrates the thermal melting curves of various TFAM deletion constructs. The data were fitted to a 2-state protein unfolding model (28). The fit returned the melting temperatures ($T_m$, midpoints of transition) of 308.9 K (HMG1), 296.1 K (HMG2), 310.9 K (TFAM) and 311.3 K (TFAM-ΔC) (Table 2). The thermal denaturation of individual HMG-box domain (HMG1 or HMG2) of TFAM is fully reversible, although $T_m$ of HMG2 is 12.8 K lower than that of HMG1. In the recently published X-ray crystal structure of HMG2 [PDB 3fgh; (23)], the hydrophobic core between helices $\alpha_1$ and $\alpha_2$ contained two amino acids (Y162 and Y200) that had multiple conformations suggesting a highly dynamic core that has not been seen in the non-sequence-specific HMG structures. The lower $T_m$ of HMG2 thus reflects this less rigid hydrophobic core that is also observed in sequence-specific transcription factors in the absence of DNA (e.g. Sox5 and SRY). Since N- and C-terminal tails (usually unstructured) of all HMG-boxes lie close together, we

**Table 2. Transition temperatures ($T_m$) of CD thermal melting curves for various TFAM deletion constructs**

| Deletion  | Up-scan melting temperature (K) | Re-scan melting temperature (K) |
|-----------|---------------------------------|---------------------------------|
| HMG1      | 308.9 ± 0.1                     | 308.4 ± 0.1                     |
| HMG2      | 296.1 ± 0.2                     | 296.8 ± 0.2                     |
| NEx-HMG2  | 302.6 ± 0.1                     | 302.0 ± 0.1                     |
| TFAM      | 310.9 ± 0.3                     | 310.5 ± 0.3                     |
| TFAM-ΔC   | 311.3 ± 0.8                     | 310.2 ± 0.5                     |

**Figure 2.** (Upper panels) Equilibrium sedimentation AUC of TFAM (5 μM) at speeds of 25 000 r.p.m. (open circle), 30 000 r.p.m. (time) and 35 000 r.p.m (open square). Experiments were conducted at 5°C in 25 mM Tris–HCl pH 7.4, 150 mM NaCl and 1 mM DTT. Data were fitted to either a single-exponential model (A) or a double-exponential model (B). Curve fits were shown as smooth lines. (Lower panels) Residuals of curve fits.
hypothesized that an extension to N-terminal end of HMG2 would help stabilizing the protein fold (Supplementary Figure S4). To test this hypothesis, we created a longer construct of HMG2 (denoted as NEx-HMG2), in which a partial linker (11 residues) was added to its N-terminus. In accordance with our hypothesis, NEx-HMG2 displayed a 2-fold higher than HMG1 and 150 mM ionic strength. Therefore, the linker region of TFAM plays a role in maintaining the overall fold, and thus stability, of the second HMG-box domain. Both TFAM and TFAM-AC was slightly more thermal stable than HMG1. Upon thermal denaturation, we noticed slight protein aggregation in TFAM and stable than HMG1. Upon thermal denaturation, 

### TFAM binds DNA co-operatively and with high affinity

Using fluorescence anisotropy, we performed solution-based DNA-binding experiments to measure the affinity of various TFAM deletion constructs to ssDNA and dsDNA (Table 3). In contrast to radioactivity-based electrophoretic mobility shift assay (EMSA) that is commonly employed to study DNA binding of TFAM qualitatively (23,25,29), fluorescence anisotropy allows quantitative measurement of DNA affinity. Three types of DNAs were tested for each protein construct: a 28-bp dsDNA consisting of TFAM-binding site on human mtDNA [MITOMAP map locus: MT-TFH (30); map position: 523–550 (31); denoted as 28-bp dsDNA herein], a 28-base ssDNA corresponding to the ‘heavy’-strand of the former dsDNA (28-base ssDNA) and a 50-bp dsDNA of random sequence (50-bp dsDNA).

The dissociation constant ($K_d$) of HMG1 for DNA was temperature-dependent, which is reminiscent of some DNA-binding proteins for instance ORF56 (32). In the case of HMG1, $K_d$ measured at three different temperatures followed the order of $37^\circ{C}<20^\circ{C}<5^\circ{C}$ (Figure 3A), despite the low $T_m$ of HMG1 ($36^\circ{C}$, Table 2). We ascribed this observation to the nature of reversible thermal denaturation of HMG1 (Figure 4A). At body temperature ($37^\circ{C}$), folded HMG1 plausibly exists in equilibrium with unfolded HMG1 and folded HMG1 is competent in DNA binding. The presence of DNA would, therefore, shift the equilibrium in favor of folded HMG1. For the purpose of comparing the contribution of each TFAM domain to DNA binding, we decided to conduct our DNA-binding experiments at physiological conditions namely $37^\circ{C}$ and 150 mM ionic strength. In general, DNA-binding data of HMG1 were fitted well to a simple 1-state binding model. $K_d$ for 28-bp dsDNA was 1.82 $\pm$ 0.06 (10$^{-6}$) M, which was $\sim$2-fold higher than $K_d$ for 28-base ssDNA (3.81 $\pm$ 0.42 (10$^{-6}$) M) and 50-bp dsDNA (3.58 $\pm$ 0.14 (10$^{-6}$) M). On the contrary, HMG2 displayed very weak DNA-binding capability (Supplementary Figure S5), corroborating findings from other research group (23). This unusual property of HMG2 is probably due to the presence of a polar residue (N163) in the position typically

### Table 3. Binding of DNAs (ssDNA or dsDNA) and p53N (residues 1–93) to various TFAM deletion constructs, measured using fluorescence anisotropy

| Construct       | $K_d$ (M) (simple one-state model)$^a$ | Hill equation$^b$ | Number of titrations repeated |
|-----------------|--------------------------------------|-------------------|-----------------------------|
|                 |                                      | $K_d$ (M)          | Hill constant               |                            |
| HMG1            |                                      |                   |                             |                            |
| 28-bp dsDNA     | 1.82 $\pm$ 0.06 (10$^{-6}$)          | 1.60 $\pm$ 0.14 (10$^{-6}$) | 1.11 $\pm$ 0.09 | 4 |
| 28-base ssDNA   | 3.81 $\pm$ 0.42 (10$^{-6}$)          | 3.33 $\pm$ 0.16 (10$^{-6}$) | 1.08 $\pm$ 0.03 | 3 |
| 50-bp dsDNA     | 3.58 $\pm$ 0.14 (10$^{-6}$)          | 2.60 $\pm$ 0.19 (10$^{-6}$) | 1.23 $\pm$ 0.05 | 3 |
| p53N            | 1.40 $\pm$ 0.18 (10$^{-5}$)          | 1.26 $\pm$ 0.21 (10$^{-5}$) | 1.06 $\pm$ 0.07 | 4 |
| HMG2            |                                      |                   |                             |                            |
| 28-bp dsDNA     | Poor fit                             | 6.76 $\pm$ 0.83 (10$^{-6}$) | 1.61 $\pm$ 0.24 | 5 |
| 28-base ssDNA   | Poor fit                             | 5.94 $\pm$ 0.57 (10$^{-6}$) | 1.76 $\pm$ 0.18 | 3 |
| 50-bp dsDNA     | Poor fit                             | 8.78 $\pm$ 0.40 (10$^{-6}$) | 2.36 $\pm$ 0.09 | 3 |
| p53N            | Poor fit                             | 2.05 $\pm$ 0.12 (10$^{-6}$) | 0.96 $\pm$ 0.10 | 4 |
| TFAM            |                                      |                   |                             |                            |
| 28-bp dsDNA     | 7.10 $\pm$ 0.40 (10$^{-6}$)          | 7.91 $\pm$ 0.31 (10$^{-6}$) | 1.22 $\pm$ 0.04 | 3 |
| 28-base ssDNA   | Poor fit                             | 11.72 $\pm$ 0.88 (10$^{-6}$) | 1.24 $\pm$ 0.06 | 3 |
| 50-bp dsDNA     | Poor fit                             | 11.63 $\pm$ 0.67 (10$^{-6}$) | 1.38 $\pm$ 0.11 | 3 |
| p53N            | 2.92 $\pm$ 0.42 (10$^{-6}$)          | 3.25 $\pm$ 0.71 (10$^{-6}$) | 0.93 $\pm$ 0.05 | 4 |

$^a$A linear drift term was added to the fitting equation.

$^b$Hill equation: $[\text{Protein}]^h/([\text{Protein}]^h + K_{d}^h)$ where $h$ is Hill constant.
occupied by a non-polar residue capable of intercalating into the minor groove of DNA. Moreover, the region known to make DNA contacts is completely absent from the N-terminus of helix α2 in HMG2 (23). When we aligned and compared TFAMs from various species (Supplementary Table S3), all HMG-box domains showed basic pI values except the second HMG-box domains of *Homo sapiens* (pI = 7.31) and of *Mus musculus* (pI = 6.88). DNA binding of HMG proteins usually involves electrostatic interactions, hydrogen bonds, van der Waals contacts, as well as important intercalating non-polar residues reside in either helices α1 or α2 of HMG box. Near-neutral pI of HMG2, therefore, could possibly be another explanation of its low-DNA affinity. Nevertheless, crystal structure of HMG2 clearly revealed the presence of a positively charged concave surface area that is typically involved in DNA binding of HMG boxes (23). Hence, we tested DNA binding of NEx-HMG2, the longer and more stable HMG2 construct. NEx-HMG2 showed increased DNA binding compared with HMG2 (Supplementary Figure S5A; Table 4), although affinity was 10-fold lower than that of HMG1. We reason that linker enhances DNA binding of HMG2 by providing additional DNA contacts (33,34). Thus, the second HMG-box domain of TFAM is a potential DNA binder with its DNA-binding capacity being modulated by the linker region. A smaller isoform of TFAM mRNA was found in most human tissue with a 96-base deletion. Since the fifth exon is identical to the 96 bases skipped in this shorter mRNA, this isoform is concluded to be generated by alternative splicing (11). Interestingly, the region of deletion corresponds to the second HMG-box domain (E148–Q179, 32 residues; Figure 1A) that showed weak DNA binding.

To fit data from TFAM and TFAM-ΔC experiments, 1-state-binding model could not be applied. These data, however, were fitted well to the Hill equation indicating co-operativity in DNA binding (Figures 3B and 3C). Compared with HMG1, TFAM showed significantly higher affinity to 28-bp dsDNA (27-fold), 28-base ssDNA (64-fold) and 50-bp dsDNA (41-fold). When the C-terminal tail was truncated, DNA-binding affinity was reduced ~1.2–2-folds. This result is consistent with findings from EMSA [2.2-fold reduction; (23)]. It is worth a note that positive co-operativity was reduced in all three DNAs tested, after removal of the TFAM C-tail (1.61→1.22 in 28-bp dsDNA, 1.76→1.24 in 28-base ssDNA and 2.36→1.38 in 50-bp dsDNA). Since the C-tail modulates self-association of TFAM, we postulate that TFAM binds as a dimer on DNA resulting in a more positive co-operativity (Figure 4B). The exact mechanism and interface of TFAM self-association, of course, requires further studies. TFAM dimerization upon DNA binding was also supported by evidences from non-contact atomic force microscopy (26) and from EMSA (23). Mutational analysis of TFAM revealed that the C-tail is important for specific DNA recognition and essential for transcriptional activation (35,36). The C-tail facilitates TFAM dimerization on DNA.
et al. reported that C-tail truncation decreased DNA-binding activity of TFAM by three orders of magnitude using surface plasmon resonance (SPR) kinetic analysis (38). We have observed non-specific interaction between TFAM and sensor chip (data not shown). The discrepancy between our data and those reported by Ohgaki et al. is likely contributed by various factors including different DNAs used (sequence and length), non-specific TFAM–sensor chip interaction and fitting model (1:1 Langmuir binding model was used in the report from Ohgaki et al.).

TFAM contains two tryptophan residues in each HMG-box domain (W88 and W107 in the first domain, W189 and W218 in the second domain). Of these, W88, W189 and W218 are strictly conserved among TFAMs from various species (Supplementary Figure S6). Figure 5A shows the intrinsic fluorescence spectra of TFAM, in the absence and presence of DNA. Excitation at 280 nm gave an emission maximum at 344 nm, implying that some of these tryptophan residues are solvent exposed. Upon binding DNA, emission maxima were blue-shifted to $\sim$338 nm and the fluorescence emissions were reduced 31–52%. Thus, these conserved tryptophan residues are involved in stabilizing protein–nucleic acid complexes, plausibly via stacking interactions with the nucleotide bases of DNA or intercalating into DNA grooves. The binding of TFAM to DNA has also an effect on its secondary structure, as evaluated by UV CD spectropolarimetry (Figure 5B). The CD-spectrum of TFAM–DNA complex shows a decrease in optical ellipticity in the spectral region 208–235 nm, compared with that of DNA-free TFAM. The decrease is indicative of formation of additional $\alpha$-helical region or type-II $\beta$-turn. We did not observe significant change in the CD spectrum $>240$ nm, which would otherwise indicate DNA conformational change from B-DNA to either A-DNA or Z-DNA. Although HMG-box is widely recognized to induce DNA deformations (bending and unwinding) upon DNA binding, this feature is not apparent in our CD measurements using 28-bp dsDNA (Supplementary Table S1). The magnitude of DNA deformation induced by HMG-box is likely DNA-sequence or DNA-length dependent.

Taken together, our solution-based DNA-binding data conform to the reported biological roles of TFAM as a transcriptional activator (preference towards sequence-specific DNA) and a mtDNA-packaging factor (high affinity for non-sequence-specific DNA).

**TFAM binds p53N**

Tumor suppressor p53 has previously been demonstrated to interact with DNA-binding proteins. Salient examples include human mitochondrial single-stranded DNA-binding protein [HmtSSB; (13)], replication protein A [RPA; (39)] and transcriptional co-activator PC4 (40),

Table 4. Binding of 5'-fluorescein-labeled DNAs (linear or bulged) to various TFAM deletion constructs, measured using fluorescence anisotropy

| TFAM       | $K_d$ (M) (simple one-state model) | Hill equation$^\text{a}$ | Number of titrations repeated |
|------------|----------------------------------|--------------------------|-----------------------------|
| Normal DNA-S | Poor fit                         | 4.13 ± 0.59 (10$^{-7}$)  | 1.26 ± 0.14                        | 5                              |
| Bulged DNA-S | Poor fit                         | 1.64 ± 0.17 (10$^{-7}$)  | 1.24 ± 0.12                        | 3                              |
| Normal DNA-L | Poor fit                         | 2.14 ± 0.33 (10$^{-8}$)  | 2.04 ± 0.19                        | 3                              |
| Bulged DNA-L | Poor fit                         | 3.15 ± 1.08 (10$^{-8}$)  | 1.85 ± 0.21                        | 3                              |
| NEp-HMG2    |                                  |                          |                              |
| Normal DNA-L |                                | 2.92 ± 0.34 (10$^{-5}$)  | 2.69 ± 0.32 (10$^{-5}$)  | 1.09 ± 0.16                        | 3                              |
| Normal DNA-L | Estimated to be $>$40 mM         |                          |                              |

$^\text{a}$Hill equation: $[\text{Protein}]^h/(K_h + [\text{Protein}]^h)$ where $h$ is Hill constant.

*Table 4. Binding of 5'-fluorescein-labeled DNAs (linear or bulged) to various TFAM deletion constructs, measured using fluorescence anisotropy.*

*Figure 4. (A) Proposed DNA-binding mechanism of HMG1. Folded HMG1 exists in equilibrium with unfolded HMG1 and folded HMG1 is competent in DNA binding. The presence of DNA would shift the equilibrium in favor of folded HMG1. (B) Schematic DNA-binding models of TFAM and TFAM-ΔC. The C-tail modulates self-association of TFAM and promotes TFAM dimer formation upon DNA binding. This accounts for more positive co-operativity in DNA binding of TFAM, in comparison to TFAM-ΔC.*

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via N-terminal transactivation domain of p53. It has also been reported that p53 interacts with HMG proteins, including HMGB1 (41) and TFAM (8). On the basis of these previous findings, we hypothesized that TFAM-binding site on p53 resides within its transactivation domain. By using fluorescence anisotropy, we assayed TFAM for binding to an AlexaFluor 546-labeled p53N (residues 1–93) at physiological ionic strength and our hypothesis was indeed verified (Figure 6). The binding data of all protein constructs tested were fitted well to a simple 1-state binding model (Table 3), and affinity towards p53N followed the order of TFAM (1.95 μM) > TFAM-ΔC (2.92 μM) > HMGB1 (14 μM). To localize the binding interface on p53N, we recorded 2D 1H,15N-HSQC NMR spectra of 15N-labeled p53N (100 mM) in the absence and presence of unlabeled HMGB1 (100 μM). In Figure 7, free 15N-labeled p53N spectrum (red) was overlaid with the HMGB1-bound spectrum (blue). p53N interacts clearly with HMGB1 through an extended interface. Large chemical shift perturbations were mainly localized in three regions: region I (residues 18–26), region II (residues 30–38) and region III (residues 40–56). The p53 transactivation domain is subdivided into two loosely defined sub-domains (42), TAD1 (residues 1–40) and TAD2 (residues 41–61). We found that HMGB1 interacts simultaneously with TAD1 and TAD2, similar to HmtSSB (13) and transcriptional co-activator p300 (43). Both sub-domains contain a Φ-X-X-Φ motif (where Φ = hydrophobic amino acid and X = any amino acid) that has been shown to be important in many protein–protein interactions [Supplementary Figure S7; (44)]. Our NMR experiment demonstrated that p53–HMGB1 interaction involves these two motifs (F19-W23 in TAD1 and I50-F54 in TAD2), signifying the involvement of hydrophobic interaction in stabilizing p53–HMGB1 complex. Further, HMGB1-binding site identified in our NMR study coincides with p53N regions that were known to form helical structures upon complex formation [i.e. helix 1 (S15-P27), helix 2 (D41-M44) and helix 3 (P47-T55); Supplementary Figure S7] (39,45). We believe that electrostatic interactions contribute substantially to p53N–HMGB1 complex formation, in view of acidic nature of p53N (pI = 3.47) and basic nature of HMGB1 (pI = 9.99).
Yoshida et al. reported that interaction between TFAM and p53 required either one of the two HMG-box domains and residues 363–376 of p53 (8), based on pull-down assays. We, therefore, assayed TFAM for binding to a fluorescein-labeled p53C (residues 362–393) at physiological ionic strength, using fluorescent anisotropy. All protein constructs tested (TFAM, HMG2, NEx-HMG2) showed weak binding to p53C (Supplementary Figure S5B). We ascribe this low affinity to basic nature of p53C (pI = 9.61) and suggest that the C-tail of p53 is possibly a secondary binding site of TFAM. Protein partners having two different binding sites on p53 have previously been reported. These include S100 [binds transactivation and tetramerization domains of p53; (46)] and Mdm2 [binds transactivation and core domains of p53; (47)].

Both TFAM and p53 bind distorted DNA preferentially

TFAM has previously been demonstrated to bind preferentially to oxidatively damaged DNA (e.g. 8-oxo-7,8-dihydroguanine), cisplatinated-DNA and DNA lesions induced by chemical carcinogens (e.g. N-acetoxy-acetylaminofluorene), using EMSA assays (48,49). To further investigate this preference quantitatively, we used fluorescence anisotropy to study binding of TFAM to two different DNAs. One of these was a linear DNA with 12 bp (denoted as Normal-DNA-S). The other was a bulged DNA containing a dA2 bulge, with its sequence being identical to the previous one (Bulged-DNA-S). We chose to study bulged DNA because it is structurally analogous to UV-induced pyrimidine dimers (naturally occurring DNA lesions) and cisplatinated-DNA (adducts induced in vivo by anticancer drug cisplatin). Both pyrimidine dimer and cisplatination induce DNA bends of magnitude comparable with the bulge-induced bends (50,51). Therefore, the study of bulged DNA is biologically relevant. As shown in Figure 8A and Table 4, TFAM bound distorted DNA preferentially. $K_d$ for Bulged-DNA-S (164 nM) was 2.5-fold lower, compared with that for Normal-DNA-S (413 nM). Nevertheless, this preference was not evident when we used DNAs that were four times longer in length (Normal-DNA-L and Bulged-DNA-L). Interestingly, p53 displayed the same preference (Figure 8B), although $K_d$ of p53 for short DNA was much higher. Taken together, p53–TFAM interaction may play a significant role in DNA damage and repair. Further, our data provided direct evidence that TFAM can indeed bind DNA as short as 12 bp, in agreement with estimation from Pellegrini et al. that TFAM was present at a ratio of one molecule per ~10–12 bp of mtDNA (52). This estimation was based on quantitative western and Southern blot analyses, using protein–DNA isolated from mouse liver mitochondria.

NMR backbone resonance assignments of HMG1 and HMG2

For the purpose of mapping p53N-binding site on HMG-box domain, we assigned the backbone resonances in the NMR spectra of both HMG1 and HMG2. $^{13}$C, $^{15}$N-labeled HMG1 and $^{13}$C, $^{15}$N-labeled HMG2 samples provided well-dispersed NMR spectra at 15°C and 5°C, respectively (Figure 9). Low temperatures were chosen to populate natively folded HMG1 and HMG2, in view of their low $T_m$ (Supplementary Figure S3; Table 2). This allowed determination of >85% of the backbone resonance assignments from HMG1 (97%, Supplementary Table S4) and HMG2 (87%, Supplementary Table S5), as described in ‘Materials and Methods’ section. Even though the spectrum of HMG2 was recorded at 5°C, we noticed a large number of sharp overlapping resonances in the random coil region of the spectrum ($^1$H, 8.0–8.5 ppm). Therefore, HMG2 is partially structured in the DNA-free protein, which explains its lower thermal stability (Supplementary Figure S3; Table 2). The intrinsic disorder in DNA-free HMG-box was also documented for SRY box (53) and LEF-1 box (54). To localize p53N-binding site, we acquired 2D $^1$H,$^{15}$N-HSQC spectra of $^{13}$C,$^{15}$N-labeled HMG1 (100 μM) in the absence and presence of unlabeled p53N (100 μM). In Figure 10A, we presented the overlay spectra
of free HMG1 (red) and p53N-bound HMG1 (blue). Large chemical shift perturbations were mapped to regions of high-helical propensity, as predicted by PSIPRED (Supplementary Figure S7). It appears that p53N interacts extensively with helices α1 and α2 (Supplementary Figure S7). The $^{1}H$, $^{15}N$-HSQC spectrum of HMG1–DNA complex (1:1 ratio, 100 μM each) is shown in Figure 10B (green). Drastic chemical shift changes have occurred for the HMG1 resonances, reflecting either extensive DNA-binding interface or conformational change. When we used a ratio of 2.5 HMG1 to 1 DNA, most peaks disappeared in the $^{1}H$, $^{15}N$-HSQC spectrum (Supplementary Figure S8). This is a common phenomenon when high-molecular weight complexes are formed. In other words, there were more than one HMG-box bound to a single DNA molecule, when HMG-box was present in excess (a ‘pigeonhole principle’ applies in this case). Of note, we observed perturbation of tryptophan side chain resonances upon DNA binding ($^{1}HN$ 10–12 ppm, indole sidechain region). This indicates that tryptophan side chains were positioned in a different environment, corroborating our intrinsic fluorescence measurement (Figure 5A).

Comparison of HMG1 homology model with HMG2 structure

Using NMR chemical shift data of HMG1, we created a homology model of this HMG-box domain (see ‘Materials and Methods’ section). HMG1 adopts canonical HMG-box fold and consists of an extended region (residues 50–55) and three helices (residues 56–71, 77–90, 93–116) that are approximately orthogonal to one another (Figure 11A). The N-terminal extended region packs against the C-terminal helix α3. The core, formed at the intersection of the three helices, comprises residues Phe60, Trp88 and Tyr99 which are surrounded by Tyr57, Ile84, Leu91 and Lys96. In addition, the orientation of helix α1 toward helix α2 is stabilized by several hydrophobic contacts formed by residues Leu65, Phe68 and Leu80 (Figure 12A). Structural comparison of HMG1 model and HMG2 structure (3fgh) reveals that these two HMG-box domains are very similar and superpose with a root mean square deviation of 2.1 Å (Figure 11A). Most of the residues forming the hydrophobic core, at the intersection between the three helices, are invariant. The only exception is that Phe60 and Ile84 of HMG1 are replaced by Tyr165 and Val185 in HMG2, respectively (Figure 12). Despite the global similarity, several differences between HMG1 and HMG2 structures are apparent. These include the region connecting helices α1 and α2, and the length of helix α1 (Figure 11B). HMG2 contains a four-residue deletion resulting in a shorter helix α1. None of the residues forming contacts between helices α1 and α2 are conserved. Leu65, Phe68 and Leu80 of HMG1 are replaced by Phe170, Ala173 and Lys181 in HMG2, respectively. Thus, the orientation of helix α1 towards helix α2 in HMG2 is determined by the hydrophobic contacts between the aliphatic portion of Lys181 and the side-chains of Phe170 and Ala173. Although the large-to-small substitution of Phe68→Ala173 is partly compensated by the substitution of Leu65→Phe170, this rearrangement causes a small displacement of helix α1 relative to the axes of helices α2 and α3. The electrostatic potential surface comparison of HMG1 and HMG2 shows that the overall charge of the DNA-binding interface is preserved (Figure 13). The displacement of helix α1 in HMG2, however, widens the concave DNA-binding interface and could be a potential reason for the weaker DNA-binding property of this domain (Supplementary Figure SS; Table 4). Modeling of the N-terminal linker of NEx-HMG2 (11 residues) suggests that some of the hydrophobic residues in this region might have a role in the stabilization of HMG-box, as observed experimentally (Supplementary
Figure S3). In particular, Leu152 could form a hydrophobic contact with Trp218 and thus orient the main-chain carbonyl group of Gly153 in a position suitable for hydrogen bond formation (Supplementary Figure S4). Since this 11-residue linker is highly positively charged, we would expect that the linker contributes to DNA anchoring, as observed in other HMG-box containing proteins (33,34).
DISCUSSION

Structure–function relationships of TFAM

TFAM is a multi-functional protein, involved in various aspects of mtDNA integrity maintenance: transcription, replication, nucleoid formation, DNA protection, DNA repair and damage sensing (3). In part, its functional diversity is a result of TFAM’s domain architecture (Figure 1A). TFAM is constructed by two tandem HMG-box domains, which are connected by a basic 36-residue linker. To the C-terminus of the second HMG-box domain, a 27-residue tail is attached, that is also rich in basic amino acid residues. In order to investigate the DNA-binding propensity and function of each domain of TFAM, we adopted a ‘divide-and-conquer’ strategy by creating a series of TFAM deletion constructs as depicted in Figure 1A. Using equilibrium sedimentation AUC (Table 1), we showed that HMG1 and HMG2 were monomers in solution. On the contrary, TFAM exists as monomer–dimer in equilibrium (Figure 2) and its self-association is modulated by the basic C-tail. This finding was validated by 1D 1H-NMR spectra of TFAM and TFAM–ΔC (data not shown). The anomalous elution profile of TFAM in size-exclusion chromatography

Figure 10. (A) 2D 1H,15N-HSQC NMR spectra of 13C,15N-labeled HMG1 (100 μM) in the absence (red) and presence of 100 μM of p53N (blue). (B) 2D 1H,15N-HSQC NMR spectra of 13C,15N-labeled HMG1 (100 μM) in the absence (red) and presence of 100 μM of DNA (green). All spectra were recorded at 15 °C in 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM DTT and 5% (v/v) D2O.

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Supplementary Figure S1) is contributed mainly by HMG1. This ‘high mobility’ is a consequence of asymmetric nature of TFAM and interaction between highly charged TFAM and chromatographic material. TFAM is thermally unstable, with a $T_m$ close to body temperature (37.7°C) determined from CD spectropolarimetry (Supplementary Figure S3; Table 2). HMG1 displayed similar $T_m$ (35.7°C), while HMG2 showed substantially lower $T_m$ (23.0°C). The thermal instability of DNA-free HMG2 well reflects its less rigid hydrophobic core (23) and its intrinsic disorder that we observed in 2D $^1$H,$^15$N-HSQC spectrum of HMG2 (Figure 9B). The linker region enhances stability of HMG2, possibly by providing additional contacts with C-end of HMG2 and thus

Figure 11. Comparison of HMG-box domains (HMG1 and HMG2) of TFAM. (A) Stereo views of structural superposition of HMG2 (3fgh, red) overlaid with a bundle of 25 HMG1 models (black). Images were generated using PYMOL. (B) Structure-based sequence alignment of HMG1 and HMG2. Secondary structure elements were shown above the protein sequences. Invariant residues between HMG1 and HMG2 were highlighted in yellow.

Figure 12. (A) Hydrophobic core of HMG1 model, with Y57, F60, I84, W88, L91, K96 and Y99 indicated. (B) Hydrophobic core of HMG2 (3fgh) with Y162, Y165, V185, W189, L192, K197 and Y200 indicated. Both images were generated using PYMOL.
Despite its low affinity and a preference towards sequence-specific DNA, TFAM binds DNA co-operatively with nanomolar binding experiments using fluorescent anisotropy, configuration (23). From our solution-based DNA-maintaining the global fold of three helices in an L-shape red. (Figure S5A), likely by providing additional DNA contacts (33,34). It is worth noting that the highly conserved tryptophan residues (Supplementary Figure S6) are involved in DNA binding, as judged from intrinsic fluorescence measurement (Figure 5A) and 2D 1H,15N-HSQC spectrum of HMG1–DNA complex (Figure 10B). Finally, DNA binding induces conformational change of TFAM (Figure 5B). The decrease in optical ellipticity at spectral region 208–235 nm indicates formation of additional z-helical region or type-II β-turn, possibly at intrinsic disorder region resides within HMG2 or linker region.

**Biological implications of TFAM–p53 interaction**

Tumor suppressor p53 is involved in a multitude of cellular functions including cell-cycle arrest, apoptosis, senescence, DNA damage and repair (55). For the first time, we showed that TFAM interacts primarily with N-terminal transactivation domain of p53 (p53N, residues 1–93, Figures 6, 7 and 10A). C-terminal regulatory domain of p53 (p53C, residues 362–393) provides a secondary binding site for TFAM (Supplementary Figure S5B).

p53N is inherently devoid of any tertiary structure. Nevertheless, a pre-existing amphipathic helix (T18-L26) and two nascent turns (turn I: M40-M44 and turn II: D48-W53, both weakly amphipathic) were found in unbound p53N, using heteronuclear multi-dimensional NMR spectroscopy (45). These local secondary structures are selectively formed by functionally critical and positionally conserved hydrophobic residues (hydrophobic antennae, e.g. F19, L22, W23 and L26 in p53N) present in several acidic transactivation domains (AADs) (45). Such local structures are general features of AADs and may represent ‘specificity determinants’ that are important for transcriptional activity (56). We speculate that the structural transitions associated with p53N upon TFAM binding involve both (i) tightening of the pre-existing helix into a stable helix and (ii) conversion of the two nascent turns (turns I and II) into an amphipathic helix. The later coil-to-helix transition is either due to structural restraints imposed by TFAM or induced by hydrophobic cores of HMG-boxes. The fact that the overall helical content of several AADs increases upon addition of hydrophobic solvents that might mimic the hydrophobic environment of a target protein supports such a possibility (45). In p53N upon TFAM binding, we picture formation of two helices (S15-L32 and Q38-D57) with a symmetrical linker (S33-S37) between them. This would also explain the chemical shift perturbations we observed in p53N (Figure 7; Supplementary Figure S7). We believe that p53N docks into the positively charged DNA-binding cleft of HMG-box (Figure 13), mimicking single-stranded DNA structurally (39,40). Amphipathic helices of p53N could plausibly orient in a way that hydrophilic sides are exposed to solvent and hydrophobic sides are buried. In this orientation, HMG-box–p53 complex is stabilized by electrostatic interactions of the negatively charged side chains of p53N and the positively charged side chains of HMG-box, as well as hydrophobic interactions between the buried surface of p53N helix.

**Figure 13.** Electrostatic surface potential plots of HMG1 model and HMG2 (3fgh), generated with PYMOL. Regions of positive potential were colored in blue, and regions of negative potential were colored in red. (A) Front views showing DNA-binding interfaces. (B) Back views.
(I50, W53, F54) and the hydrophobic core of HMG-box (Y57, F60, W88). Therefore, HMG-box represents a structural entity endowing dual affinity (i) for acidic, amphipathic helices and (ii) for DNAs. The aforementioned speculations would certainly require experimental validations, e.g. by solving the crystal structure of HMG-box–p53 complex.

Similar to RPA–p53 interaction (57), TFAM–p53 interaction could possibly involve two distinct areas of p53 (p53N and p53C, Figure 6 and Supplementary Figure S5B) simultaneously. This is likely facilitated by the presence of DNA. In the absence of DNA, p53 displays an elongated cross-shaped structure with a pair of loosely coupled core domain dimers at the ends, which are accessible for binding to DNA or protein partners (58). The core domains in this ‘open conformation’ can close around a DNA to form a compact complex, resulting in a ‘close conformation’ (58). Further, DNA induces TFAM dimerization that is modulated by TFAM C-terminal tail (Figure 4B). In a possibly compact TFAM–p53–DNA ternary complex, one would envisage the feasibility of ‘two-point interaction’.

We believe that TFAM–p53 interaction is biologically relevant. The naturally oxidative environment of mitochondria and lack of an efficient DNA repair mechanism render mtDNA highly susceptible to mutations, and these mutations are frequently associated with a variety of human diseases (1). Since both TFAM and p53 binds preferentially distorted DNA (Figure 8, Table 4), TFAM–p53 interaction is implicated in DNA damage and repair. In this same vein, we recently reported that HmtSSB (a mtDNA packaging factor and sub-unit of mtDNA replisome) binds to p53N via an extended binding interface, with dissociation constant of 12.7 ±0.7µM (13). HmtSSB enhances intrinsic 3'-5' exonuclease activity of p53 (59), particularly in hydrolysing 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) present at 3'-end of DNA (13). Our data suggest that p53 is involved in maintaining integrity of mitochondrial genome, in line with findings from other group (60). Remarkably, p53 null mouse and p53 knockdown human primary fibroblasts were shown to exhibit mtDNA depletion, decreased mitochondrial mass and membrane potential, reduced protein level of both mitochondrial membrane proteins, RNA, or even mtDNA (66). The voltage-dependant anion channel (VDAC, also termed mitochondrial porin) contains two cysteines and two methionines, in total four potential targets for platinum binding. VDAC has been shown to form cisplatin/protein adducts, resulting in permeability alteration of mitochondrial membrane (66). Likewise, cisplatin could possibly change the permeability of nuclear envelope that was noticeable in Supplementary Figure S9. Since p53 was up-regulated after drug treatment, p53 observed in perinuclear mitochondria might be a consequence of passive diffusion (Supplementary Figure S9). Remarkably, it has been demonstrated that TFAM overexpression and impairment of its mitochondrial targeting could result in nuclear accumulation of this protein (24). A putative bipartite nuclear localization sequence (two clusters of positively charged residues separated by 11 amino acids) was found in HMG1. Nuclear TFAM was found to confer cytoprotection against chemotherapeutic drugs e.g. etoposide, camptothecin and cisplatin. Mitochondrial localization of p53 and nuclear accumulation of TFAM raise the possibility that p53 and TFAM were examples of ‘eclipsed distribution’ (67). Upon DNA damage, protein traffic is likely bi-directional (p53: nuclei → mitochondria, TFAM: mitochondria → nuclei).

**Protein traffic upon DNA damage**

As a predominantly nuclear protein, p53 lacks MTS. Nevertheless, mitochondrial localization of p53 was observed after cisplatin treatment [8] and in present study; Supplementary Figure S9]. The mechanism by which p53 is transported into mitochondria remains, however, unclear. Being an important chemotherapeutics, cisplatin is best recognized as a DNA damaging agent and the cytotoxicity of cisplatin has generally been attributed to its ability to form inter- and intra-strand DNA crosslink. While most investigations of cisplatin pharmacology focussed on cisplatin/nuclear DNA adducts, only 1% of intracellular platinum is bound to nuclear DNA. The remaining majority of the intracellular drug is available for targeting nucleophilic sites on other molecules, including phospholipids, cytosolic, cytoskeletal and membrane proteins, RNA, or even mtDNA (66). The voltage-dependant anion channel (VDAC, also termed mitochondrial porin) contains two cysteines and two methionines, in total four potential targets for platinum binding. VDAC has been shown to form cisplatin/protein adducts, resulting in permeability alteration of mitochondrial membrane (66). Likewise, cisplatin could possibly change the permeability of nuclear envelope that was noticeable in Supplementary Figure S9. Since p53 was up-regulated after drug treatment, p53 observed in perinuclear mitochondria might be a consequence of passive diffusion (Supplementary Figure S9). Remarkably, it has been demonstrated that TFAM overexpression and impairment of its mitochondrial targeting could result in nuclear accumulation of this protein (24). A putative bipartite nuclear localization sequence (two clusters of positively charged residues separated by 11 amino acids) was found in HMG1. Nuclear TFAM was found to confer cytoprotection against chemotherapeutic drugs e.g. etoposide, camptothecin and cisplatin. Mitochondrial localization of p53 and nuclear accumulation of TFAM raise the possibility that p53 and TFAM were examples of ‘eclipsed distribution’ (67). Upon DNA damage, protein traffic is likely bi-directional (p53: nuclei → mitochondria, TFAM: mitochondria → nuclei).

**CONCLUSION**

In the present study, we characterized interaction between TFAM and p53. Biophysical measurements, in conjunction with cellular biology methods, provide a systematic
platform to study the physical and functional interactions between these two proteins in vitro. The biological relevance of this binding in vivo would require more in-depth investigations, in view of the complex multi-functional roles of both TFAM and p53.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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