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Structural features of human histone acetyltransferase p300 and its complex with p53

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ABSTRACT

The protein p300 is a multifunctional transcriptional coactivator that plays pivotal role in several cellular functions. Although structures of several domains have been solved in isolation, the structures of full-length protein (p300 FL) or its complexes with transcription activators are completely unknown. Herein, we applied atomic force microscopy to visualize p300 FL. We found that it is almost prolate ellipsoidal in shape, having several bulges. We further identified the functionally significant N-terminal and C-terminal regions, by applying domain-specific antibodies and found that they are located near one end and centre of the molecule, respectively. Importantly, we have visualized the complex between p300 FL and tumor suppressor protein p53. The relevance of these data in understanding dynamics of p300 during acetylation and transcription will be mentioned.

Structured summary of protein interactions:
p300 and **p53** bind by atomic force microscopy (View interaction)

1. Introduction

The p300 protein is a transcriptional coactivator [1], which plays a key role in cellular processes like proliferation, cell-cycle regulation, apoptosis, differentiation and DNA damage response [2,3]. The p300 possesses intrinsic histone acetyltransferase (HAT) activity which modifies chromatin during transcription and other DNA template phenomenon [4,5]. Being a transcriptional coactivator, it interacts with a large number of transcription factors and RNA polymerase II transcription machinery [2,6]. Despite its central role in gene expression, the crystal structure of p300 FL has not been reported yet. Recently, the cocrystal structure of p300 HAT domain with its specific, synthetic inhibitor Lys-CoA has been determined [7], and the limited trypsin proteolysis and SERS spectral analysis of p300 FL have provided some information on overall domain organization [7,8]. In this work, we have obtained information for the first time on the tertiary structure of p300 FL by applying AFM, which is a high-resolution local probe imaging method for obtaining information on single biomolecules [9]. AFM can provide direct 3D information on biomolecules of any size. Therefore, in absence of a crystal structure and due to the large size (300 kD) of p300 FL, AFM could be an attractive strategy to exploit.

There are several domains in p300 that bind transcription factors and one of the most well-studied transcription factors that recruits p300 is p53. The p53 binds DNA as a tetramer [10] and it has been shown that phosphorylation of p53 enhances its affinity towards several p300 domains distributed throughout the structure [11]. It has been suggested that all four molecules of p53 in a tetramer interact with p300 during complex formation, presumably with different domains [12]. However, no conclusive proof has been offered yet. This complexity suggests that in absence of atomically resolved structure of p300 FL, even molecular level resolution could shed light on the function of this important molecule. In this study, apart from visualizing the p300 FL protein, we have performed experiments to identify the location of the functionally important C-terminal and N-terminal regions of p300 FL and to obtain an idea about the domain structure. Moreover, we have located the position of binding of p53 onto p300 FL by using a p53 mutant which contains a point mutation by glutamic acid at threonine 18. We chose this particular mutant, since it is reported that glutamic acid mimics phosphorylated threonine residue [13] and phosphorylated threonine 18 of p53 binds most tightly with p300 [11]. There are some reports [12,14] where p53 binding to p300 is investigated using specific domains of either p300 or

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p53. Here, we have studied the binding using the two full-length protein molecules instead of the domains.

2. Materials and methods

2.1. p300 FL purification

p300 FL was purified from recombinant baculovirus-infected Sf21 cells as a His_6-tagged protein through nickel-nitrilotriacetic acid affinity column (Qiagen) as described previously [8].

2.2. Preparation of p300 FL solution

The p300 FL solution was freshly prepared in 10 mM Tris, 10% glycerol, 200 mM NaCl, 50 μl protease inhibitor cocktail (0.1% NP40, 2 mM β-ME, 2 mM PMSF, 50 μg/ml aprotinin, 50 μg/ml leupeptin) at pH 7.5 prior to each experiment.

2.3. Preparation of His-Tag mAb, IgG-AP, C-terminal specific antibody and N-terminal specific antibody solutions

The respective stock solution was suitably diluted with an appropriate buffer immediately before use. (See Supplementary materials) for details.

2.4. Preparation of p53 mutant (T18E)

Site-directed mutagenesis was performed using wild type flag tagged p53 plasmid by PCR-based site-directed mutagenesis kit (Agilent Technologies). Sequencing by dideoxy method showed that desired mutation was incorporated within the plasmid. Details of protein expression and purification are provided in Supplementary materials.

2.5. Preparation of p300 FL-antibody and p300 FL-p53 mutant complexes

In order to form p300-antibody complexes, 0.4 μl p300 (10 nM) and 0.6 μl antibody solutions (4.8 μg/ml His-Tag mAb or 4.0 μg/ml IgG-AP/C-terminal specific antibody/N-terminal specific antibody) were mixed and incubated at 37 °C for 10 min in dark. For p300 FL–p53 complex formation, 2 μl of each of 10 nM p300 FL and 10 nM p53 mutant solutions were taken. Complex formation reaction was carried out in a buffer containing 25 mM Tris–HCl, 150 mM NaCl, 20 μM $ZnCl_2$, 2.5 mM β-ME and 10% glycerol of pH 7.5 at 27 °C for 15 min in dark.

2.6. AFM sample preparation for imaging p300 FL

0.5 μl of 5 nM p300 solution was deposited onto freshly cleaved muscovite mica (ICR & Sons Pvt. Ltd., India), kept for 15 min, followed by gentle wash with 2 ml (250 μl × 8) Milli-Q water, and dried using gentle stream of nitrogen gas.

For *in situ* (i.e., in fluid) imaging of p300 FL, gold(111) on mica substrate (Phasis, Switzerland) was cleaned by flame-annealing and immersed into 0.5 mM ethanolic solution of 3-mercaptopropionic acid (3-MPA) for 1 h followed by washing with 2 ml (500 μl × 4) filtered ethanol and 2 ml (500 μl × 4) Milli-Q water, and drying with a stream of nitrogen gas. Then 3-MPA modified gold substrate was immersed into freshly prepared aqueous solution of 75 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and 15 mM N-hydroxysuccinimide (NHS), kept for 30 min, followed by washing with 2 ml (500 μl × 4) Milli-Q water. Then the 3-MPA, NHS, EDC modified gold substrate was dried with a stream of nitrogen gas. The p300 solution (50 pM) was deposited onto modified gold substrate and incubated for 10 min. The sample surface was gently washed with 2 ml (250 μl × 8) Milli-Q water.

2.7. AFM sample preparation for imaging p300 FL-antibody and p300 FL-p53 mutant complexes

1.0 μl of the p300 FL – antibody complex or the p300 FL – p53 mutant complex solution was deposited onto freshly cleaved mica and kept for 15 min, then washed with 2 ml (250 μl × 8) Milli-Q water and dried using gentle stream of nitrogen gas.

2.8. AFM data acquisition and analysis

AFM imaging was performed using PicoLE AFM equipment (Agilent Corp., USA) with 10 × 10 μm scanner at room temperature (24 ± 1.0 °C) in the intermittent contact mode (acoustic alternating current or AAC mode). Standard rectangular silicon cantilevers (μmasch, Estonia) of force constant 3.5–27.5 N/m were used for ambient (i.e., in air) imaging. The cantilever oscillation frequency was 171–202 kHz. For *in situ* imaging, SNL-10 cantilevers (Veeco) of force constant 0.24 N/m were used. The cantilever oscillation frequency was 40–75 kHz. The probes were cleaned in a UV-ozone cleaner (Bioforce, Nanosciences) before imaging. Amplitude set point was 85–90% of the free oscillation amplitude (7.5–8.0 V in ambient and 4.0–6.0 V in fluid). Scan speed was 0.5–2.0 lines/s. Standard data analysis procedures were adopted (see Supplementary materials).

3. Results and discussion

In this work, we applied high-resolution AFM to visualize p300 FL molecules *in situ* (i.e., in fluid) and in ambient (i.e., in air) condition. In order to identify the location of C-terminal, we employed a His-Tag specific monoclonal antibody (His-Tag mAb) that can bind to a His-Tag unit present at the C-terminal region of p300 FL, second, a secondary antibody molecule IgG-AP that can bind specifically to the primary antibody His-Tag mAb, and finally a C-terminal specific antibody. For identifying the N-terminal of p300 FL, N-terminal specific antibody molecule was applied. Binding of the tumor suppressor protein p53 to p300 FL was investigated using the p53 mutant T18E. AFM imaging experiments were carried out in intermittent contact mode to minimize tip-induced damage of the soft protein exterior.

3.1. Topographic features of p300 FL

The *in situ* AFM 3D views of individual p300 FL molecules of ellipsoidal shape with a distinct terminal bulge are shown in Fig. 1. Similar molecular shape could be frequently detected also in ambient condition. The average length of p300 FL molecules was estimated to be 141.6 ± 15.2 nm (from *in situ* measurements) and 141.2 ± 5.4 nm (from ambient measurements)[1]. While in all the cases the terminal bulge was clearly visible, greater resolution, as shown in Fig. 2(a), could be obtained in a few cases. Almost prolate ellipsoidal in shape, these molecules revealed presence of apparently three smaller bulges and one small side-protrusion (Fig. 2(b)) apart from the big bulge at one end. It is evident in the cross-section (Fig. 2(c)) that the terminal bulge is considerably bigger compared to the other bulges. The smoothness of the cross-section profile taken across the bulge (Fig. 2(d)) indicates that this bulge is condense and probably represents one single structural domain. In control

[1] The radius of curvature of the probe used was less than 10 nm which is small enough for imaging of protein molecules. Given the present situation of imaging p300 FL, which is quite a large protein, the probe employed in our study is expected to behave as a sufficiently sharp probe.

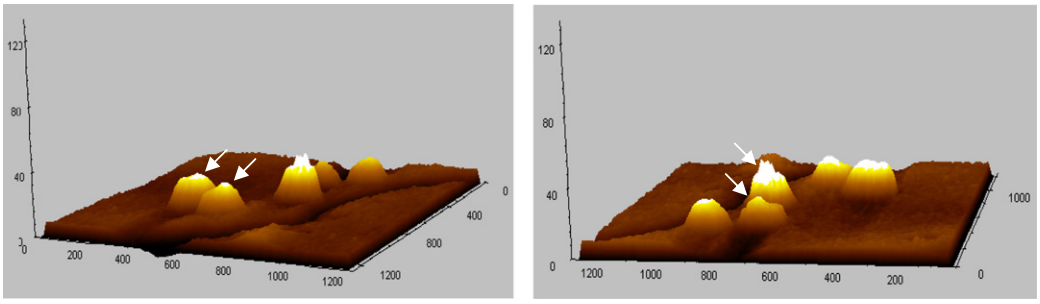

Fig. 1. *In situ* AFM topographic images (3D views) of covalently immobilized p300 molecules on NHS–EDC modified gold(111) surface (X, Y axes in nm and Z axis in Å). Imaging was carried out in a buffer solution [10 mM Tris, 200 mM NaCl, 1 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonylfluoride (PMSF) at pH 7.5]. The terminal bulge is highlighted with arrow mark.

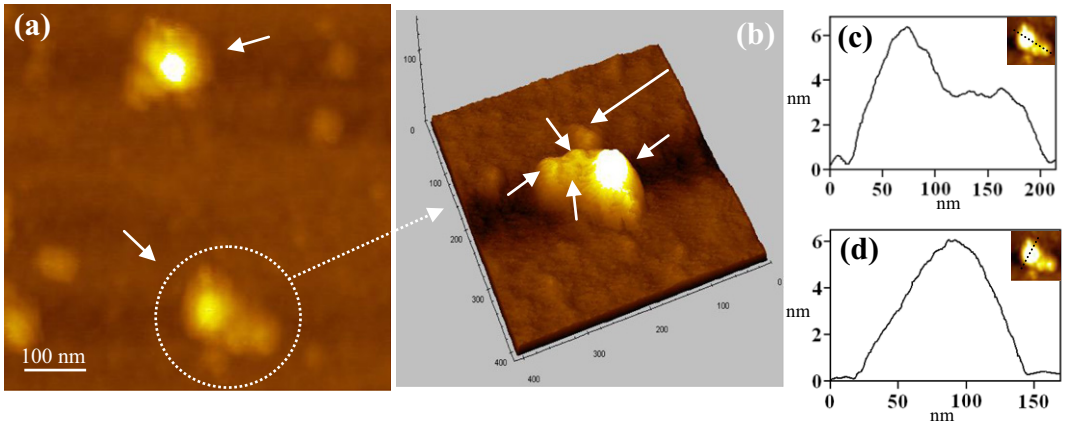

Fig. 2. (a) AFM topograph of p300 FL molecules (marked by arrows) on mica surface in ambient condition, Z-range: 0–11.2 nm; (b) magnified 3D view of one p300 molecule (X, Y axes in nm and Z axis in Å); (c) cross-section along long axis and (d) cross-section across the big condense bulge. The 3D view in (b) reveals presence of apparently four bulges (shown by short arrows) in the main body of the protein, and a protrusion (shown by long arrow).

experiments, no p300 FL-like feature could be found (Fig. 1 in Supplementary material). It has been shown earlier that when low concentration of the proteins are applied and the protein molecules are adsorbed in single isolated condition, the proteins of non-spherical shapes tend to adsorb having their long axes parallel to the surface [15,16]. Precisely this has been the observation in case of p300 FL adsorption in each of our experiments leading to reproducible observation of the ellipsoidal shape of p300 FL.

For visualization of the p300 FL in ambient condition, the p300 molecules were adsorbed on bare mica surface. But for imaging in fluid, the p300 molecules had to be covalently anchored on the surface so that they were not desorbed from surface and get attached to the AFM tip resulting in imaging difficulties. The covalent anchoring of the full-length p300 molecules was achieved on 3-MPA modified gold(111) surface with the help of NHS, EDC, where the lysine residues at the protein surface could form covalent amide links with the carboxylic end of 3-MPA [17]. In this case, the protein molecule is not directly exposed to the surface but it sits on a layer of 3-MPA. For both the situations, i.e., on bare mica surface and on the 3-MPA modified gold(111) surface, the dimension, shape and the major structural features of full-length p300 molecule remained same indicating that in both the cases, the molecule sit on the surface in similar fashion and that there was no major detrimental effect of direct adsorption on the molecular structure of p300. Earlier in several studies using AFM and STM (scanning tunneling microscopy), reasonable level of similarities between the X-ray/NMR structure of a protein molecule and its AFM/STM image characteristics was shown [18,19]. This indicates that the gross structural features are maintained on surface if the protein molecules are carefully immobilized.

3.2. The C-terminal and the N-terminal of p300 FL are located at the centre and at the terminal bulge position, respectively

In order to obtain further structural information on p300 FL, a His-Tag specific monoclonal antibody (His-Tag mAb) that can bind to a His-Tag unit introduced at the C-terminal region of p300 FL was employed. The complexes appeared mostly ellipsoidal in shape and about 151.6 ± 12.5 nm in length (Fig. 3(a)). In order to confirm the position of binding of His-Tag mAb to p300 FL, a secondary antibody molecule IgG-AP that would bind specifically to the primary antibody His-Tag mAb, was also employed [20]. The ternary complex (p300 FL– His-Tag mAb – IgG-AP) as shown in Fig. 3(b) was about 151.3 ± 13.0 nm in length. Further evidence for identification of the C-terminal region of p300 FL was still sought after since there was a possibility that His-Tag mAb could bind at a location near C-terminal and not precisely at C-terminal due to dangling disposition of His-Tag around its attachment point. We therefore applied a p300 FL C-terminal specific antibody for explicit identification of the C-terminal location. The complexes between p300 FL and this antibody were elongated in shape (Fig. 3(c)), having an average length of 151.1 ± 12.6 nm. Lastly, as a natural query whether the N-terminal region could also be detected, a p300 FL N-terminal specific antibody was employed. From the AFM topographs of p300 FL – p300 FL N-terminal specific antibody complex, it is evident that this antibody binds at a completely

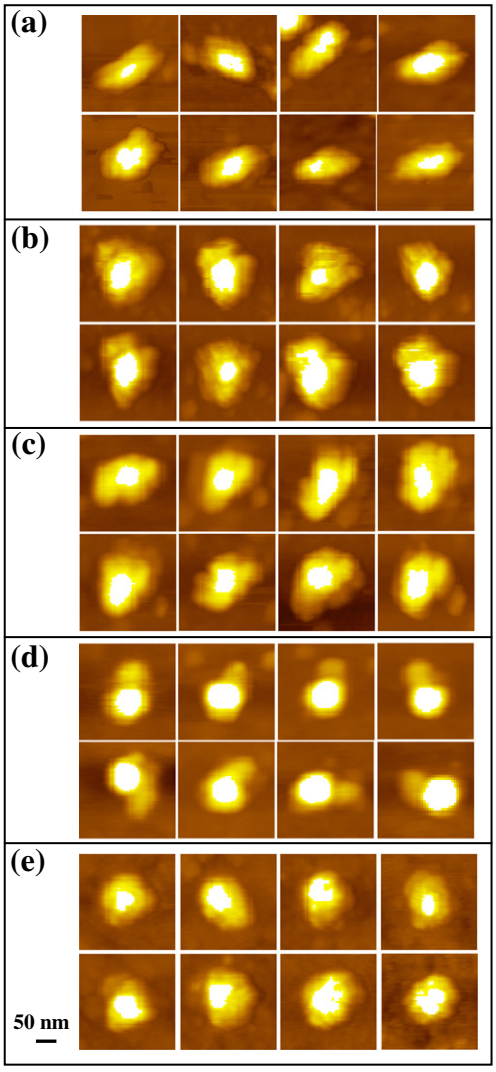

Fig. 3. AFM topographic images of the complex (a) p300 FL - His-Tag mAb, (b) p300 FL - His-Tag mAb – IgG-AP, (c) p300 FL – p300 FL C-terminal specific antibody, (d) p300 FL – p300 FL N-terminal specific antibody, (e) p300 FL – p53 mutant (T18E). Z-range: (a) 0–11.0 nm, (b) 0–15.5 nm, (c) 0–12.5 nm and (d) 0–13.0 nm, (e) 0–12.0 nm.

different region of p300 FL compared to the location for His-Tag mAb/C-terminal specific antibody binding (Fig. 3(d)). The average length of these complexes was estimated as 151.2 ± 13.4 nm. All the antibody molecules were characterized by AFM prior to complex formation step (see Supplementary material). The antibodies looked spherical in shape, and were of sizes 35.1 ± 8.3 nm (His-Tag mAb), 38.5 ± 9.9 nm (IgG-AP), 39.8 ± 13.4 nm (C-terminal specific antibody) and 38.5 ± 8.7 nm (N-terminal specific antibody).

While one large terminal hump and two small humps were observed in the cross-section profile of p300 FL (Fig. 4(a)), a smooth cross-section was observed in case of p300 FL–His-tag mAb complex (Fig. 4(b)). Observation of such a cross-section is possible only if the antibody binds at the central region of p300 FL, as depicted in the schematic diagram in Fig. 4(b). A dotted line profile, representing the path of the AFM tip, can be drawn along the contour of the schematic diagram. Here we consider the fact that the AFM tip is not sharp enough to resolve small curvatures on the protein contour. A close similarity between this dotted line profile and the cross-section along the long axis of the complex could be observed, indicating that the schematic diagram represents the p300 FL–antibody complex architecture reasonably well. The relatively

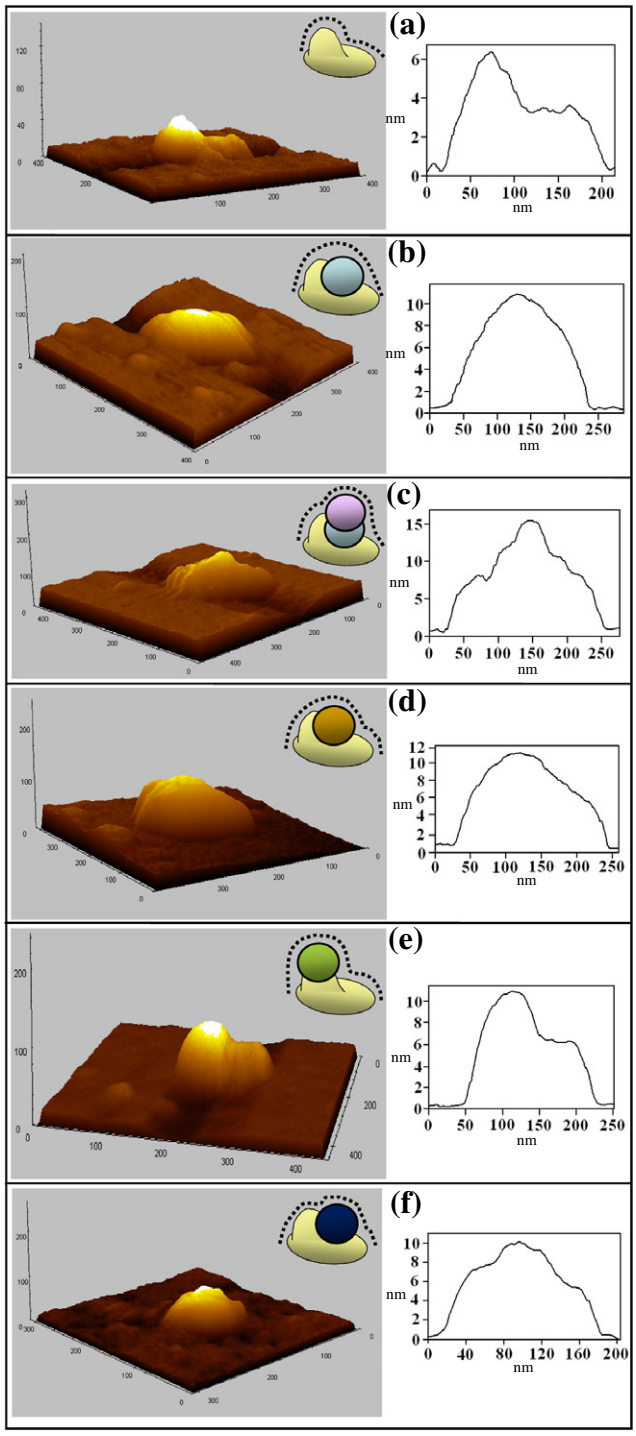

Fig. 4. Complexes formed between p300 FL and different antibody molecules. From left, are shown 3D views (X, Y axes in nm and Z axis in Å) and schematic drawings of the complexes along with a dotted line drawn along the contour of schematic as a representation of the path of the AFM tip, and cross-sections along the long axes of the complexes. Along the rows, are shown (a) p300 FL, (b) p300 FL – His-Tag mAb complex (blue ball indicates antibody positioning), (c) p300 FL – His-Tag mAb – IgG-AP complex (blue ball and pink ball indicates His-Tag mAb and IgG-AP, respectively), (d) p300 FL – p300 FL C-terminal specific antibody complex (the brown ball indicates antibody positioning), (e) p300 FL – p300 FL N-terminal specific antibody complex (green ball indicates antibody positioning), (f) p300 FL – p53 mutant (T18E) complex (dark blue ball indicates p53 positioning). For the representative image galleries and the cross-section galleries, see Supplementary material.

greater height value of the complex compared to p300 FL (Table 1) is compatible with binding of the antibody molecule and earlier re-

Table 1
Comparison between height and length values of p300 FL and p300 FL-antibody/p53 complexes.

Molecule/complex	Height (nm)	Length (nm)
Free p300 FL	3.3 ± 0.1 (central region) 6.4 ± 0.6 (terminal bulge)	141.6 ± 15.2 (*in situ* condition) 141.2 ± 5.4 (ambient condition)
p300 FL – His-Tag mAb	10.2 ± 0.9	151.6 ± 12.5
p300 FL – His-Tag mAb – IgG-AP	13.5 ± 2.5	151.3 ± 13.0
p300 FL – p300 FL C-terminal specific antibody	11.6 ± 1.7	151.1 ± 12.6
p300 FL – p300 FL N-terminal specific antibody	9.6 ± 2.0	151.2 ± 13.4
p300 FL – p53 mutant (T18E)	11.3 ± 1.2	129.5 ± 9.2

ports that antibody binding to an antigen can be elicited from AFM height value increase [21]. The p300 FL – His-Tag mAb – IgG-AP complex clearly revealed a symmetric disposition (see cross-section in Fig. 4(c)) quite like that for the p300 FL – His-Tag mAb complex (Fig. 4(b)), except for a protruded central region and a greater height value (Table 1). Formation of such a contour is possible only if the secondary antibody molecule binds on top of the primary antibody molecule (see the schematic diagram in Fig. 4(c)). These findings support the fact that the primary antibody binds to the central region of p300 FL, and therefore the C-terminal is located at the central region of p300 FL. Further confirmatory evidence in support of this proposition could be found from the observation of an almost symmetric cross-section profile of the p300 FL – C-terminal specific antibody complex (Fig. 4(d)). It is clearly indicated in the cross-section that the antibody binds at the central region of p300 FL. The greater height value of the complex compared to that of the free p300 FL (Table 1) also corresponds well with antibody binding. These observations (Figs. 4(a)–(d)) therefore collectively confirm that the C-terminal of p300 FL is centrally located.

In case of the p300 FL – N-terminal specific antibody complex, the cross-section profile was asymmetric (Fig. 4(e)), and quite similar to the cross-section of p300 FL, except for an increased height value (Table 1), plausibly as a result of antibody binding at the terminal bulge region. This result not only reflects the fact that the N-terminal of p300 FL is located at the terminal bulge region of the protein, but also confirms that the previously described antibody (His-Tag mAb/C-terminal specific antibody) binding interactions are specific in nature.

3.3. The p53 binds at the central region of p300 FL

After obtaining an idea about the location of the C-terminal and N-terminal of p300 FL, we investigated the binding of the tumor suppressor protein p53 to p300 FL. We chose a p53 mutant[2] where threonine 18 is replaced with the phosphomimetic amino acid, glutamic acid [13], since it has been shown that threonine 18 phosphorylated p53 binds most tightly with p300 [11]. The complex between p300 FL and p53 mutant were found to be elongated in shape (Fig. 3(e)) having an average length of 129.5 ± 9.2 nm, which is less than the length of free p300 FL. This probably suggests that while forming the complex with p53, the full-length p300 molecule folded to some extent, as predicted by Teufel et al. that the p300 FL 'wraps around p53' for complex formation [12], giving rise to the shorter length of the complex. The cross-section profile of the p300 FL – p53 complex indicates that p53 binds at the central region of p300 FL. We have shown earlier in section 3.2 that the C-terminal end of p300 FL is also centrally located. These observations agree well to the observation made by Gu et al., who showed that p53 binds to the C-terminal region of CREB-binding protein (CBP), which shares extensive homology to the p300 FL [6]. The p53 protein is normally a tetramer and hence contains four activation domains. Recently, a model has been proposed in which four domains bind to Taz1, Taz2, IHD and Ibid domains of p300 simultaneously [12]. Although the present data cannot directly confirm or reject such a hypothesis[3], it is important to note that the presence of N- and C-terminal regions near the central portion of the prolate ellipsoid suggests that all four domain could exist spatially close near the central portion of the molecule and hence capable of binding all four domains simultaneously. Interestingly, the bound p53 molecule can also be located in this region, indirectly supporting the model.

4. Conclusion

In conclusion, p300 FL molecules were found to be nearly prolate ellipsoidal in shape, having multiple bulges of different sizes – distributed throughout the structure, which all together could represent the different structural domains of p300 FL. We have identified the locations of C-terminal and N-terminal regions of p300 FL at the central part and at the terminal bulge part, respectively, by employing C-terminal specific and N-terminal specific antibody molecules. In addition, we have located the p53 binding region on p300 FL, which appears to be at the C-terminal region of p300 FL molecule. Furthermore, the size of the p300 HAT domain (residues 1284–1517), which is the largest domain of p300 FL, is such that it appears to be a part of the largest bulge observed in the AFM topographs. Here, it is tempting to presume that the non-bulge region, which may represent the rest of the p300 domains, is necessary for most of the protein–protein interactions in the p300 molecule, and which would further fold back upon a factor (protein) binding for translating the binding message to the catalytic (HAT) domain of the p300. The presence of the single large bulge in our study supports the monomeric nature of p300 FL as suggested for the p300 HAT domain [7]. Interestingly, our model also provides a physical understanding of the trans-nature of the autoacetylation of the p300 [22] where the large bulge part (HAT domain) can be easily available to the other p300 molecule. Thus, it appears that the arrangement of the protein domains in p300 is not linear/sequential. The approach taken here could provide further insight into domain relationships, if more domain-specific monoclonal antibodies can be raised. This study may also help to develop p300 as a model protein molecule to study inter-domain interaction within a single protein molecule.

[2] The p53 mutant was characterized by AFM (see Supplementary material). These molecules were spherical in shape having width distributions 24–26 nm and 34–36 nm.

[3] Though it is really interesting to correlate the height values with the binding of monomeric, dimeric or tetrameric p53, it could be oversimplified in our situation. The molecular weight of an antibody molecule is ~150 kD and the monomeric p53 should have a molecular weight of ~53 kD. We found that the total height value of p300 FL – His-Tag mAb complex and p300 FL – p300 FL C-terminal specific antibody complex (where one antibody molecule is bound at the central region of p300) is close to the height value of p300 FL – p53 complex where p53 is bound at the central portion of p300 (see Table 1). From this comparison, we can suggest that it is not the monomeric p53 that is binding with p300 because in that case it will be a 53 kD protein (p53) binding with p300 that can't give rise to a height value similar to that of 150 kD antibody binding. Hence, it could be either dimeric (i.e., ~100 kD) or tetrameric (i.e., ~200 kD) p53 that is binding with p300 FL.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.09.012.

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