Functional Imaging — Using SPM to Visualize Enzymes at Submolecular Resolution

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INTRODUCTION

There is great interest in unraveling action mechanisms of key enzymes in biological processes. In many cases, insight on such molecular events can be derived from conventional biophysical analyses of isolated enzymes and their substrates or protein partners. For example, members of the matrix metalloproteinases (MMP) family have been implicated in numerous aspects of the migration of inflammatory and cancer cells through connective tissues, not only by degrading extracellular matrix (ECM) components but also by processing various soluble mediators, promoting many disease states.[1] MMP-9, also known as gelatinase B, is a prototypical target in inflammatory diseases, because of its tissue-damaging roles and inflammation-promoting processing of soluble proteins.[2] Despite its importance, the full length structure of this multidomain enzyme is not known.

By exploiting modern computational and synthetic techniques, the link between structure and function can be applied to probe mechanisms of action. Revealing molecular mechanisms is critical in establishing therapeutic treatments, and to further understand biological processes. X-ray diffraction of crystalline samples, combined with computational analysis, can provide detailed maps of the primary, secondary, and tertiary structure for even complicated proteins. For the case of pro-MMP-9, structural characterization has been hampered by the fact that the molecule cannot be crystallized for x-ray structural analysis. This is because the "linker" domain separating two terminal domains of the active protein lends a high degree of freedom to the molecular orientations. In fact, this linker domain is significantly longer than in any other members of the MMP family. Insights into the structure of such flexible linker may be obtained by small-angle x-ray scattering (SAXS). This technique can deliver structural information at a resolution of several nanometers, even on isolated protein molecules randomly dispersed in solution. For monodisperse solutions of non-interacting particles, the scattering intensity curves reveal different single-molecule conformations, which can be fit to deduce such possible structures. Since the solution of the scattering profile is not unique, additional structural input that constrains the molecular size and shape is required for convergence.

Scanning probe microscopy (SPM) has established an important niche in the field of biological characterization, due to several advantages that are not universally present in alternate techniques. These include the performance of high-resolution imaging in native environments, the ability to produce structural characterization on systems lacking long-range order, and the nondestructive character of the technique. Still, acquiring reproducible and high-resolution SPM images of small biological molecules is not a straightforward task. The small size of such proteins (several nanometers across) pushes the limits of accurate SPM imaging. A quick review of the SPM literature shows that the highest resolution is typically obtained on large objects: either long strands such as DNA, collagen, etc., or ordered membranes built up from the molecule of interest. These structures provide stability due to good immobilization of the protein. On the other hand, sharp

images of single, isolated proteins are not a routine matter. This is because an individual protein is often not as tightly bound to a surface and lacks the cooperative effects gained even by the nonspecific binding existing in the larger assemblies. Attempts to use specific links to bind the molecule of interest to a flat, solid substrate carry the danger of forcing the protein into a constrained geometry, which can mask the structure-function relationships being sought.

In this application note, a protocol is demonstrated for characterizing the flexible structure of pro-MMP-9 using SPM. The results of these microscopic studies were used as input and fine-tuning for SAXS models. The combination of imaging and scattering techniques, together with hydrodynamic studies, yielded a consistent model for this enzyme showing clearly two terminal domains joined by a flexible linker. In addition, SPM provided rather unique information on possible protein conformations adapted by the enzyme upon its interaction with surfaces. Comparison with a mutant lacking this linker revealed the important role of the linker domain.

SUBSTRATES FOR ENZYME BINDING

Smooth single-crystal silicon or mica are ideal substrates on which to image small molecules, due to the ease with which featureless, ultra-flat surfaces can be formed. In addition to the physical characteristics, we require that the substrates bind the protein securely without altering or confining its natural shape. Amine-modification of substrates has been shown to result in an accessible covalent bonding through the glutaraldehyde linkage, without destroying the smoothness of the underlying substrate.[3, 4] In this work, we made use of amine-modified silicon surfaces (see figure 1) as the substrate of choice. The plasma process employed did not alter the smoothness of the surface, and furthermore allowed control of the surface amine concentration.

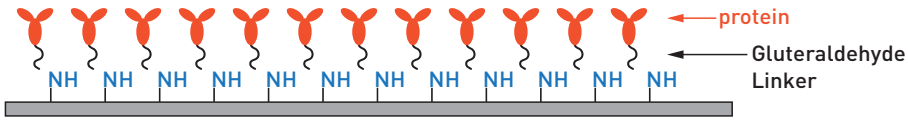

Figure 1. Schematic of aminized surface showing how the protein binds through the gluteraldehyde linker.

Ideally, high-resolution images acquired under buffer solution would provide the closest approximation to the molecule in its natural state. Unfortunately, the fully hydrated molecule could not be imaged sharply, as seen in figure 2A. This phenomenon may arise because the loose binding of the protein to the surface possibly still allows some degree of motion, or it could be related to the fact that the hydrated sample is softer and easier to distort under tip pressure. Highly dehydrated samples, achieved by placing the sample in a vacuum desiccator overnight, likewise resulted in poor quality images (see figure 2B).

Best image quality was obtained under, ambient conditions whereby the sample was rinsed and excess water removed by 2 to 3 minutes of gentle nitrogen flow. Thus, sample volumes of 100 microliters, containing 0.1 milligrams per milliliter monodisperse solution of pro-MMP9ΔOG mutant or wild-type pro-MMP-9, fractionated to contain the monomeric form, were incubated on the amine-modified silicon dies for 3 hours. The samples were rinsed gently with 2×200 microliters buffer, followed by 5x200 microliters Milli-Q water, and finally dried under the stream of nitrogen. This procedure likely leaves a thin hydration layer on the sample and is hence termed "semi-dry mode." The importance of this hydration layer in atomic force microscopy (AFM) imaging is suggested to result in images representative of the fully hydrated molecule.[5, 6]

In any event, when working with a dried sample, it is necessary that it be clean of debris and dried salt deposited from the accompanying buffer solution. The amine modification provided sufficient binding of the protein so that buffer salts could be readily rinsed away, without removing the enzyme from the surface (see figure 2C). XPS measurements verified the presence of the amine at a surface concentration of 4.7%. At this level of amine concentration, and

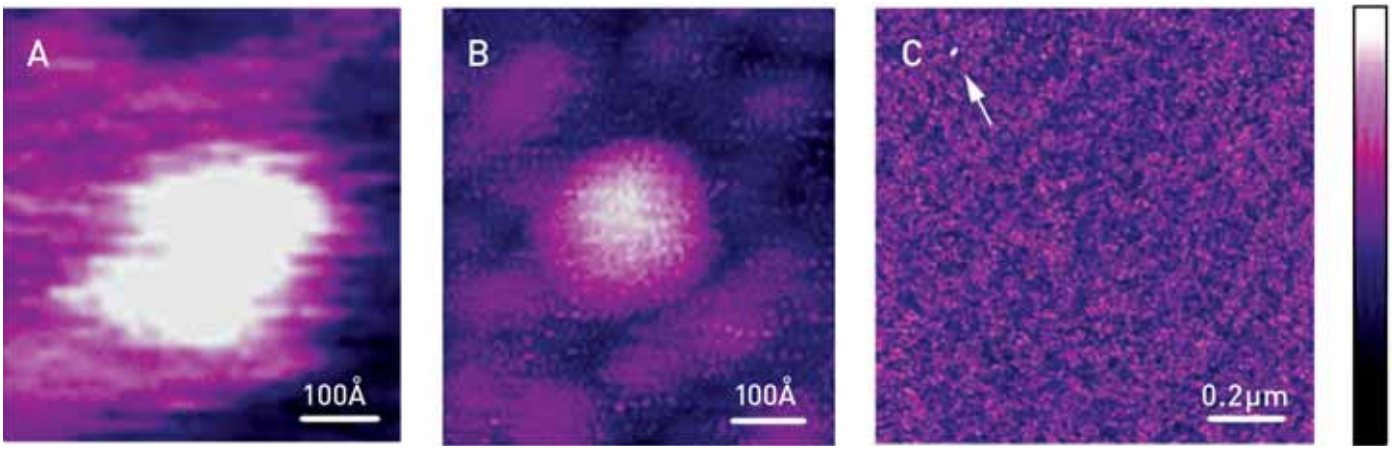

Figure 2. AFM images of pro-MMP-9. (A) Wild-type pro-MMP-9 under buffer solution, using oxide-sharpened silicon nitride tip. (B) Dessicated sample of the wild-type enzyme scanned in ambient conditions with spike tip. (C) Blank sample subjected to the same immobilization procedure but without applying the enzyme (spike tip). The arrow indicates the only particle observed on 1x1µm^2 scan. Height scale of the color bar ranges from 0 to 50Å (dark to light). (Reprinted with permission from reference 7.)

considering the molecular footprint, there are statistically no more than four such bonds per protein molecule, which furthermore would not form simultaneously as the molecule approaches the substrate. From this we conclude that the binding itself cannot significantly constrain the protein conformation. Horseradish peroxidase assays showed that increasing the amine surface coverage beyond this level did not enhance binding.

AFM IMAGING AND ANALYSIS

Samples were imaged in air or in buffer using TappingMode™. In order to minimize the amount of force applied, the amplitude set point was adjusted to the maximum value that gave a stable trace. High-resolution images of biological samples in air were obtained using diamond-like carbon "spike" tips with a rated tip radius of curvature of 2 nanometers or less. These probes are only suitable for measurements on surfaces with an RMS roughness of less then 20 nanometers were used for the liquid measurements, which were performed in the standard Veeco MultiMode® AFM liquid cell. The sizes of the protein molecules were determined from cross-sectional analysis. The width values were than corrected for broadening by the tip, by subtracting the tip envelope as observed from a typical high-resolution electron microscope image.

RESULTS

The sample preparation procedure described above assured that >95% of the features observed (approximately 20 in a 1×1 square micron image) represented the enzyme. Typical images of the wild-type and mutant enzyme are shown in figure 3. The image cross-section indicates the existence of two separated protein domains, which in the model structure would be connected by the OG linker (see figure 3C). In contrast, the pro-MMP-9ΔOG mutant lacking the 64-residue OG domain exhibits a rather spherical shape with unresolved domain separation.

The overall results are summarized in the histograms of figure 4, which show the height and width distributions of the two different samples. The two enzyme species are easily distinguishable in that the spread in both width and height values for the wild type is significantly larger than for the mutant. Such differences could arise from the additional degrees of freedom lent to the wild-type structure by the OG domain, as opposed to the mutant, where the two lobes are more confined. The heterogeneity of sizes results from two main factors, the different orientations of the protein on the surface, and different protein conformations. The mutant has reduced conformational degrees of freedom because it lacks the OG domain, so in this case the spread of values stems mainly from different orientations on the surface.

These AFM results are consistent with the reported SAXS analysis, and reveal the details of the elongated multi-domain structure of pro-MMP-9. The AFM results, together with hydrodynamic measurements, placed additional constraints on the possible molecular conformations consistent with the SAXS measurements, and provided a more concise view of the molecular structure. Figure 5 shows a composite of the AFM image with the molecular model.

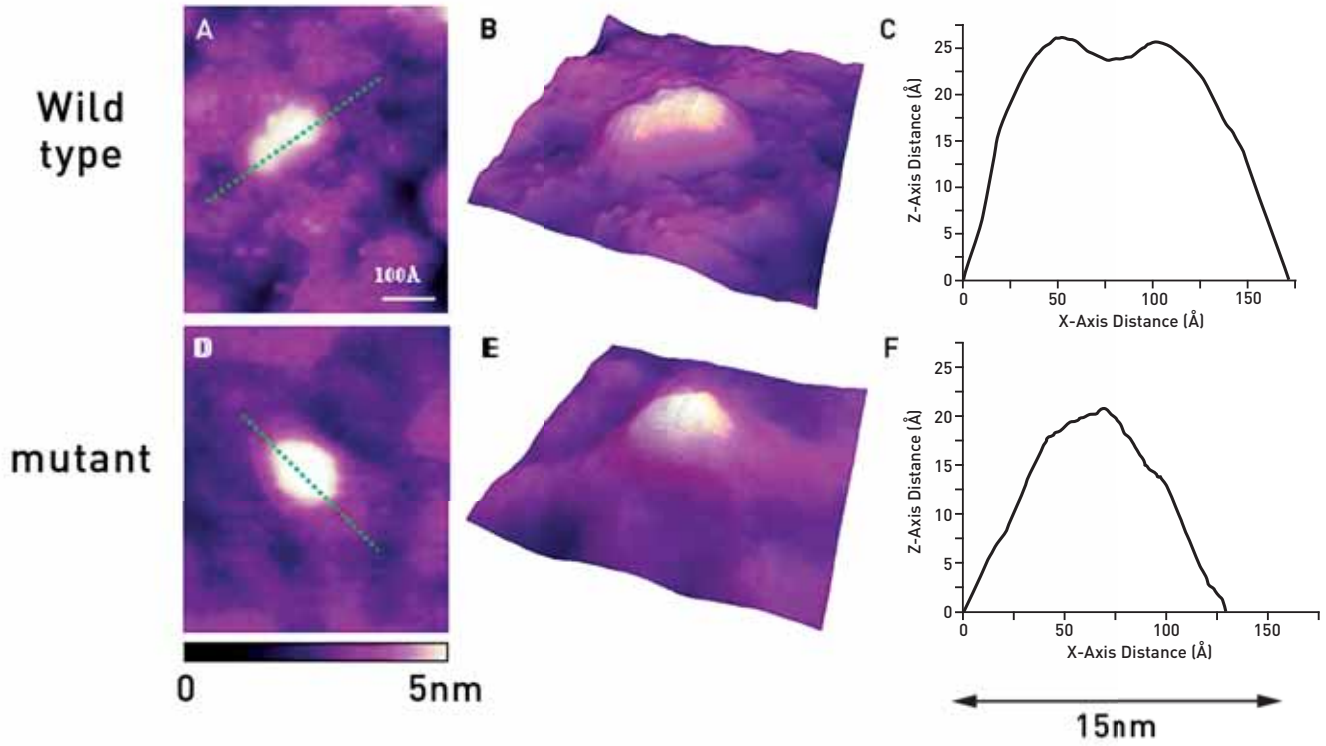

Figure 3. AFM images of wild-type (A-C) and mutated (D-F) pro-MMP-9. All scans employed a spike tip. The cross-sections in (C) and (F) represent the dotted lines in the 2D images in (A) and (D), respectively, and clearly show the difference in domain structure for the wild-type and mutant species. Height scale of the color bar ranges from 0 to 50Å (dark to ligh). (Reprinted with permission from reference 7.)

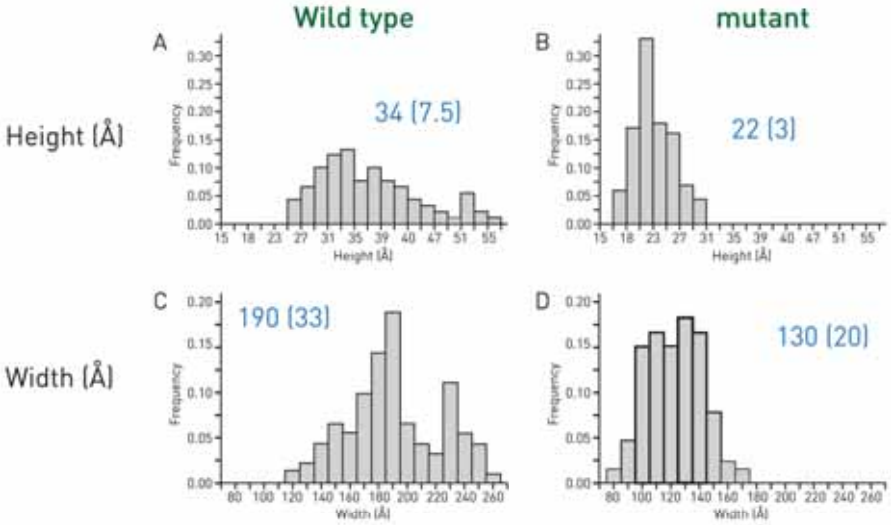

Figure 4. Height (A) and (B), width (C) and (D), and interlobe distance (E) histograms of the wild-type (left) and pro-MMP-9ΔOG (right) as measured by AFM. The y-axis of all histograms is normalized to an overall sum of one. The interlobe distribution in E shows a bimodal structure, whereby the two peaks (interlobe distances of 62Å and 79Å) agree well with the minimal (linker fully compressed) and maximal (linker fully extended) conformations as calculated by RAPPER. The separation between lobes in the mutant pro-MMP-9ΔOG could not be resolved. (Reprinted with permission from reference 7.)

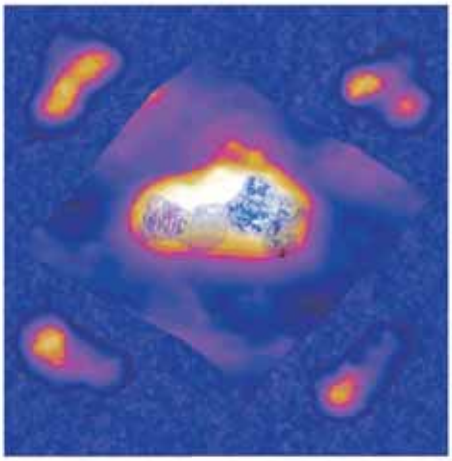

Figure 5. Molecular model derived from SAXS measurements as superimposed on the AFM image of pro-MMP-9. Surrounding are additional AFM images sampling some of the different conformations expressed on the substrate.

CONCLUSION

Enzymes are flexible moieties with structures exhibiting dynamical fluctuations over a wide range of timescales. Such inherent mobility of a protein fold was shown to be manifested in the various steps constituting the catalytic cycle. The nature of this linkage between protein structure movement and function is complex and hard to resolve by conventional experimental tools used in the fields of biochemistry and biology. Therefore, detection of protein conformation transitions in many cases remains elusive. Here, we report a novel functional imaging experimental procedure that enables the detection of the envelope structure and conformation of proteins. Notably, this procedure allows the characterization of macromolecules of relatively small size with sub-molecular detail. Specifically, for pro-MMP-9, the combination of single-molecule imaging and SAXS provided important structure-function information about the molecular character of this dynamic enzyme that could not be detected by conventional methods.[7] This information is currently being utilized for the rationalization and design of novel therapeutics.

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