Chemical Force Microscopy with Active Enzymes

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ABSTRACT The adhesion forces have been measured between an atomic force microscope tip derivatized with an active enzyme, shikimate kinase, and an ATP mimic immobilized on a gold surface. Experiments with competitive binding of other ligands in solution show that the observed adhesion forces arise predominantly from specific interactions between the immobilized enzyme and surface-bound adenine derivative. These experiments represent a step in the development of a screening methodology based upon chemical force microscopy.

INTRODUCTION

An atomic force microscope (AFM) has the sensitivity to measure forces comparable with that of a single hydrogen bond (Hoh et al., 1992). From this observation it was a small step to realize that placing appropriate molecules on the tip of an AFM probe would create a microscope with molecular, spatial, and energetic resolution. This is the chemical force microscope (CFM) that has been used to study hydrophobic interactions (Frisbie et al., 1994; Noy et al., 1995), chirality (McKendry et al., 1998), and the interactions between proteins and ligands (Florin et al., 1994; Moy et al., 1994; Lee et al., 1994). Chemical force microscopy has been proposed as a method suitable for screening large numbers of ligands in order to expedite the discovery of therapeutically useful enzyme inhibitors (Allen et al., 1996; Green et al., 1999). Here we report the first direct measurements of competitive binding interactions between an enzyme immobilized upon the tip of an AFM and two ligands, one immobilized on a surface and the second in free solution (Fig. 1). This is the first step in the development of a screening methodology based on CFM.

The enzyme used in these studies is shikimate kinase, the fifth enzyme on the shikimate pathway. It catalyses the interaction of shikimic acid with adenosine triphosphate (ATP) to form shikimate 3-phosphate and adenosine diphosphate (ADP). The enzyme is a 19-kDa monomer whose crystal structure has been solved recently (Krell et al., 1998). This enzyme is a potential antiparasitic target for which there are no known inhibitors. On storage, the enzyme dimerizes due to disulfide formation. The dimer, which maintains catalytic activity, was used throughout our studies.

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MATERIALS AND METHODS

Enzyme immobilization

The first and most important step in our AFM-based screening protocol was the immobilization of the enzyme while retaining catalytic activity. The approach used was to immobilize the enzyme onto a carboxyl-terminated self-assembled monolayer (SAM) formed on the gold-coated AFM tip using *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (EDC) coupling (Frey and Corn, 1996) of lysine side chains on the protein surface to the surface carboxyl groups. It would not be expected that this generic coupling procedure would impose any specific orientation of the protein on the surface, and so the integrity of the immobilized enzyme was confirmed by the combined use of surface plasmon resonance (SPR), AFM, and direct assay of the immobilized enzyme activity.

SPR chips functionalized with 11-mercaptoundecanoic acid were activated as their NHS esters and immediately functionalized with shikimate kinase. Unreacted ester groups were capped with a basic solution of ethanolamine hydrochloride. From the SPR sensorgram (Karlsson and Falt, 1997) it was possible to estimate the "footprint" of a single molecule of shikimate kinase on the surface. Complete derivatization of the acid thiol monolayer with shikimate kinase resulted in a change in the SPR angle of 2200 response units (Stenberg et al., 1991), corresponding to 2.2 ng/mm^2 of shikimate kinase. Based on this loading, each dimer of shikimate kinase had a footprint of 80 × 80 Å, which is in reasonable agreement with the predicted size of the enzyme based upon the crystal structure (Krell et al., 1998).

The integrity of the immobilized enzyme was further confirmed by comparison of the catalytic activity and the substrate dissociation constants. The catalytic activity of an immobilized enzyme film and a comparable amount of enzyme in free solution was measured by coupling the production of ADP to the NADH-dependent reduction of pyruvate using pyruvate kinase and lactic dehydrogenase in a double-coupled assay. The oxidation of NADH was monitored by UV spectrophotometry at 340 nm (Krell et al., 1998) The immobilized enzyme was found to retain approximately half the activity of the enzyme in free solution.

The substrate equilibrium dissociation constants K_D for ATP and shikimic acid were determined by SPR. For this experiment, the shikimate kinase was immobilized on a carboxylated dextran matrix, which permits greater loading densities. Solutions of a range of concentrations of ATP or shikimic acid were passed serially first over a flow cell containing immobilized BSA, then over a flow cell containing immobilized shikimate kinase with the BIACORE 2000 instrument (Biacore AB, Stevenage, UK) in multi-flow cell mode. A set of typical response curves (sensorgrams) is shown in Fig. 2. The complex was allowed to dissociate for 500 s, then residually bound ligand was removed using a pulse of acidic glycine. The resulting sensorgrams were corrected for bulk refractive index changes and signal drift. As both ATP and shikimic acid dissociated extremely rapidly from shikimate kinase, affinities were calculated from analysis of equilibrium binding levels at varying analyte concentrations. The dissociation

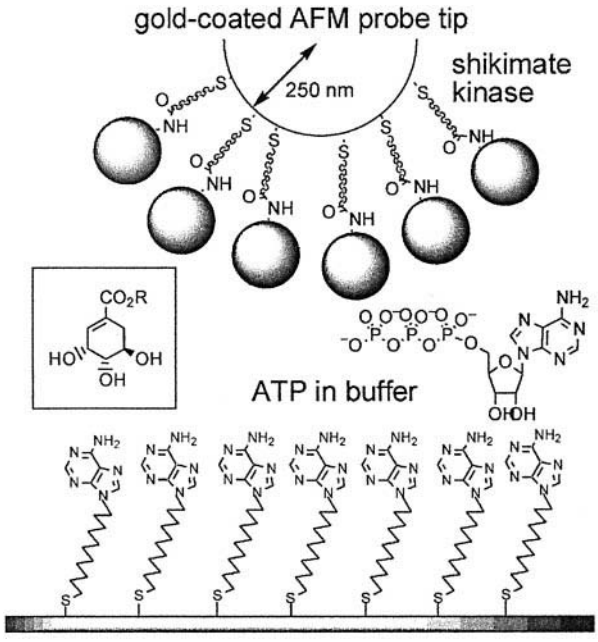

FIGURE 1 Schematic diagram of the CFM experiment, showing an enzyme bound to the AFM tip, with a substrate mimic presented as the headgroup of a self-assembled monolayer adsorbed on a gold(111) surface and competing with ligand in solution. In the experiment described in the text, the enzyme is shikimate kinase, the headgroup is adenine, and the competing ligand is ATP. The inset shows shikimic acid (R = H) or methyl shikimate (R = Me).

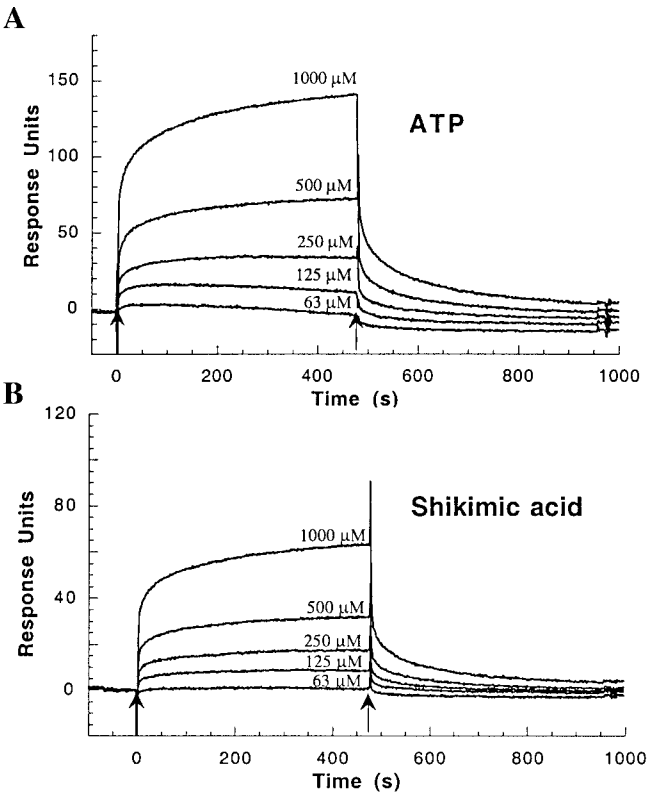

FIGURE 2 Sensorgram from the SPR experiment showing binding of ATP and shikimic acid (63–1000 μM) to 2200 RU of shikimate kinase immobilized on carboxydextran. Arrows represent start and end of substrate injection.

constants determined by this procedure were $K_D = 325 \pm 50$ μM for shikimic acid (averaged over a series of three experiments), and $K_D = 840 \pm 250$ μM for ATP. These values are close to those obtained using solution assays (shikimic acid, $K_D = 220$ μM and ATP, $K_D = 700$ μM) (Brown, 1994) implying that the immobilization has not greatly affected the enzyme's binding affinity.

Careful independent characterization of functionalized surfaces is essential in studies using AFM. In these experiments it was necessary not only to know the enzyme was on the surface, but also that the process of immobilization did not significantly modify its properties. The three independent determinations of footprint, catalytic activity, and dissociation constants have provided indirect but convincing evidence that this was the case.

As a final check on the immobilization procedure the deposition of shikimate kinase onto an NHS-activated carboxythiol SAM was studied by AFM as a function of time. Fig. 3 shows a series of images collected over a period of 2 h. Analysis of these figures showed that the surface roughness increased rapidly as enzyme was deposited in islands upon the surface. This was followed by a decrease in surface roughness as gaps in the monolayer were progressively filled. Under the conditions used in this study, a densely packed homogeneous enzyme film is formed within 2 h. The topographic z-measurements indicated the formation of a single layer of immobilized enzyme (80–100 Å), in good agreement with SPR measurements (80 Å) and the known structure. These conditions were then used to immobilize the enzyme on the AFM tip.

In order to amplify the interaction between enzyme on the tip and the molecules on the surface, a large blunt tip was used. This is different from many previous studies, where very sharp AFM tips have been used in order to study individual molecular interactions (Hinterdorfer et al., 1996). A blunt tip has the advantage of an increased contact area accommodating more copies of the enzyme, and decreased pressures being exerted on the surface species. The concomitant loss of lateral resolution is not important in this study. AFM tips were coated in 200 nm of gold and their radius of curvature, measured by SEM, was 250 nm.

Design and characterization of the functionalized surface

The experimental design involves measuring the reduction in the binding interaction between immobilized enzyme on the AFM tip and a substrate analog or inhibitor on the surface caused by molecules in solution. As there are no documented inhibitors of shikimate kinase, a substrate analog was used. Specifically, adenine was used as a mimic of the headgroup of ATP. This heterocycle was attached to the end of a long alkyl thiol and used to form a self-assembled monolayer on a gold surface (Fig. 1). This SAM should be generally useful for looking for competitive binders at the ATP binding site of any ATP-dependent enzyme.

The adenine-derivatized surface was characterized by infrared spectroscopy and contact angle measurements. Simple contact angle measurements of water on this surface gave a value of 60°, which suggested that the polar nitrogens and the aromatic ring present a moderately hydrophilic surface. The orientation of the adenine headgroup was investigated by reflection absorption infrared spectroscopy (RAIRS). From a comparison between the RAIRS spectra for the mimic immobilized on a surface and the compound in a KBr disk, it was not possible to determine the absolute orientation of the inhibitor on the surface. However, the presence of symmetric and asymmetric C–H stretches indicated that the long alkyl chain was tilted with respect to the surface normal. The absence of the N–H stretch in the RAIRS spectra suggested that the bond was oriented parallel

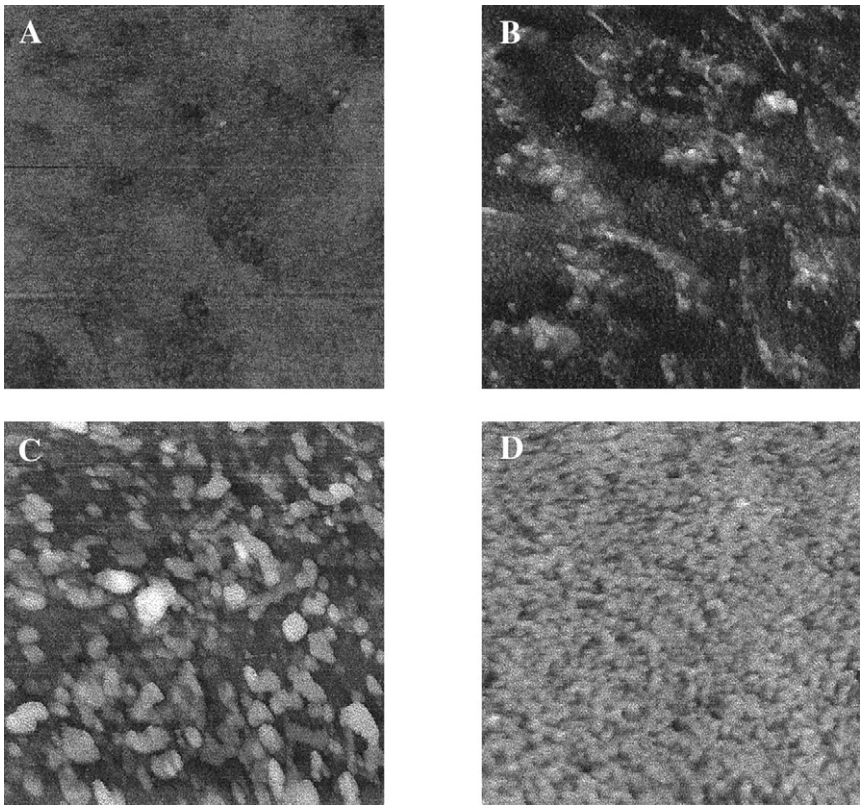

FIGURE 3 Topographic AFM images showing (*a*) a gold surface functionalized with a SAM terminating in an NHS ester, and (*b*) the same surface after incubation with shikimate kinase for 30 min, (*c*) 60 min, and (*d*) 120 min. (Images were taken under triethanolamine buffer at pH 7, scan area = 2 μm $\times$ 2 μm, a change from black to white in the image corresponds to 10 nm.)

to the surface. This implies that the chain was tilted with respect to the surface, while the adenine group was perpendicular to the surface.

AFM binding experiments

The mechanism of shikimate kinase is not known in detail. The order of binding of ATP and shikimate is not known, and there is some disagreement about whether the binding of the two substrates is independent (Brown, 1994). Therefore, a series of experiments were planned that would investigate the interaction of the enzyme with the surface, and how this was affected by competition with ATP and shikimic acid.

The adhesion force was determined by collecting 60 consecutive force-distance curves at a fixed point on the surface after the solution had equilibrated for 10–20 min. To ensure reproducibility and to test for tip damage, a further 60 adhesion force measurements were subsequently taken. The distribution of 60 adhesion force measurements between the immobilized enzyme and the adenine alkylthiol SAM is shown in Fig. 4 for measurements conducted in 100 mM triethanolamine buffer at pH 7 (the optimum conditions for shikimate kinase, and at 298 K). It is found that the average adhesion force was 14.0 $\pm$ 0.7 nN. The magnitude of the adhesion force was reduced linearly as ATP was added to the solution between the AFM tip and the surface (Fig. 5 and Table 1). The end point in this series of experiments was using 4.2 mM ATP. Under these conditions the adhesion force was 7.0 $\pm$ 0.4 nN. The magnitude of the measured adhesion forces is considerably larger than measurements observed in previous studies of protein ligand interactions (Florin et al., 1994; Moy et al., 1994; Lee et al., 1994). This is to be expected because the tips used in these experiments were made larger than those used in earlier studies to improve statistical accuracy and to reduce damage to the enzyme induced by pressure.

A series of control experiments were conducted, the results of which are summarized in Table 2. A solution of shikimate kinase was heated to 100°C for 15 min in order to denature the enzyme. A tip functionalized with the denatured form of shikimate kinase showed no adhesion to the adenine surface. Measurements with a bare silicon nitride tip yielded similar results. The addition of either shikimic acid or methyl shikimate, at comparable concentrations with those of ATP, had no significant effect upon the magnitude of the adhesion force between a tip derivatized with shikimate kinase and the adenine SAM. Likewise, addition of magnesium phosphate (4.2 mM) had no effect. This result was important because ATP is a charged molecule in which magnesium plays an important role, and we wished to test for changes caused purely by alteration of the Debye screening length for the solution and variation of surface charge.

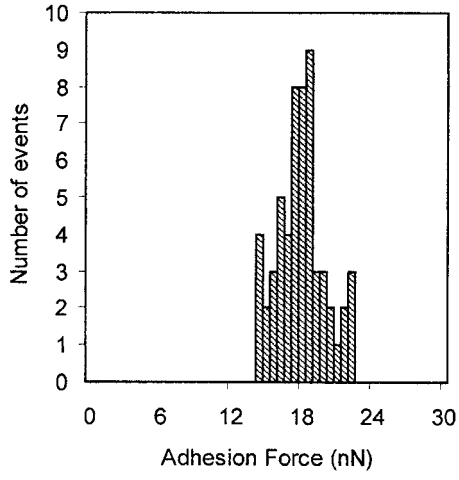

FIGURE 4 A histogram of adhesion forces between shikimate kinase on the AFM tip and the ATP mimic on the surface, measurements taken in a buffer solution.

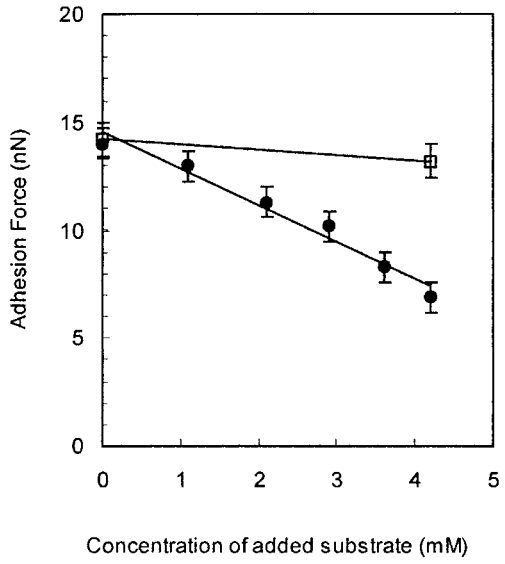

FIGURE 5 A graph showing the dependence of the adhesion force between an AFM tip derivatized with shikimate kinase and a self-assembled monolayer of an ATP mimic at various concentrations of added substrate ATP (●) and shikimate (□) in 100 mM ethanolamine buffer.

As a final test of the specificity of the interaction between the enzyme and the adenine derivative, measurements were made in different concentrations of the ionic buffer (HCl/KOH triethanolamine, pH 7 (Table 3). It was found that raising the salt concentration did not attenuate the adhesion. In fact, it actually enhanced adhesion slightly, but did not change the ATP-dependence of that adhesion. This confirms that the data cannot be explained simply by electrostatic screening.

Preparation of self-assembled monolayers on gold surfaces

Annealed gold samples were prepared on freshly cleaved mica surfaces by thermal evaporation in a Balzers UTT 400 UHV thin film preparation system. Before gold deposition the chamber was baked at 160°C for at least 12 h to ensure high-quality operational vacuum, and the mica samples were heated to 340°C. This achieved a base pressure $<10^{-7}$ mbar. Thermal evaporation took place at a rate of 10 Å/s and pressures $<10^{-7}$ mbar to produce gold films that were 1000 Å in thickness. After deposition, the gold samples were annealed at 340°C for 5 h. Samples were removed from the evaporator and immediately immersed in a 1 mM ethanolic solution of either mercaptoundecanoic acid or the adenine alkanethiol. Before use in the CFM experiment, samples were rinsed thoroughly in ethanol and dried in a stream of nitrogen.

TABLE 1 The average adhesion force between an AFM tip functionalized with shikimate kinase and a surface coated with a thiolated adenine mimic upon successive injections of ATP

Molarity of ATP (mM)	Adhesion Force (nN)
0	14.0 ± 0.7
1.1	13.0 ± 0.5
2.1	11.3 ± 0.7
2.9	10.2 ± 0.7
3.6	8.3 ± 0.8
4.2	6.9 ± 0.7

TABLE 2 The average adhesion force between a tip functionalized with shikimate kinase and a surface coated with an adenine alkanethiol SAM upon addition of identical concentrations of ATP, magnesium phosphate, methyl shikimate, and shikimic acid

Additive (4.2 mM)	Adhesion Force before Addition (nN)	Adhesion Force after Addition (nN)
ATP	14.0 ± 0.7	7.0 ± 0.4
$Mg_3(PO_4)_2$	13.0 ± 0.6	12.5 ± 0.5
Methyl shikimate	13.1 ± 0.7	12.2 ± 0.2
Shikimic acid	14.2 ± 0.8	13.2 ± 0.6

Enzyme immobilization on surfaces

Purified shikimate kinase was kindly supplied by Professor John Coggins (Department of Biochemistry, University of Glasgow) as concentrated solutions (23 mg/ml) in 50% glycerol/50% buffer. Carboxylic acid terminated SAMs were immersed in an aqueous solution containing 400 mM EDC and 100 mM NHS for 30 min. The resultant NHS ester monolayers were incubated for up to 1 h at 25°C in the enzyme solution (100 μg/ml, 300 mM sodium acetate buffer, pH 4.5). Any unreacted ester groups were capped with ethanolamine hydrochloride (1 M, pH 8.5, 10 min). After removal of the SAM from the enzyme solution, the surface was thoroughly rinsed with MilliQ water and triethanolamine HCl-KOH buffer (100 mM, pH 7.0) before use.

Surface plasmon resonance experiments

The buffer used for all experiments was PBS (100 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, pH 7.4). The BIACORE 2000 SPR instrument, CM5 sensor chips, NHS, EDC, and ethanolamine hydrochloride were obtained from Biacore AB. The sensor chips contained four flow cells of dimensions 2.4 × 5 × 0.05 mm (l × w × h) with a probing spot for the SPR signal of ~0.26 mm^2 for each flow cell.

Immobilization of shikimate kinase

Equal volumes of NHS (50 μl, 50 mM in water) and EDC (50 μl, 200 mM in water) were mixed together, then 50 μl of this solution injected at 10 μl/min across flow cell 2 of a CM5 sensor chip. This was followed immediately by an injection of shikimate kinase in acetate buffer, pH 4.5 (30 μl, 25 μg/ml), resulting in the immobilization of 2200 RU of protein. Residual NHS esters were then inactivated by an injection of ethanolamine

TABLE 3 Average adhesion force measurements between a tip functionalized with shikimate kinase and a surface coated with a thiolated adenine mimic, in different concentrations of salt buffers

Buffer Concentration (mM)	Adhesion Force (nN)
100	12.5 ± 1.4
200	13.3 ± 1.2
300	16.3 ± 1.4
400	19.6 ± 1.4
500	20.9 ± 1.4

(50 μl, 1 M, pH 8.5). In a similar manner, BSA (10 μl, 2.6 mg/ml) was immobilized on the first flow cell of a CM5 sensor chip, resulting in the immobilization of 1700 RU of protein.

Determination of dissociation constants

Solutions of ATP (63–1000 μM, 80 μl, 20 μl/min, 100 mM PBS, 1 mM $MgCl_2$, pH 7.4) were passed serially first over a flow cell containing immobilized BSA, then over a flow cell containing immobilized shikimate kinase with the BIACORE instrument in multi-flow cell mode. The complex was allowed to dissociate for 500 s, then a pulse of 10 mM glycine at pH 2 (10 μl, 20 μl/min) was injected across both surfaces to remove any residually bound ATP. Binding of shikimic acid was assayed in a similar manner. All assays were performed at 25°C in triplicate.

Data were prepared for analysis by subtracting the average response recorded 20 s before injection and adjusting the time of each injection to zero. Data from the flow cell containing BSA were subtracted from corresponding data obtained from the shikimate kinase-containing flow cell to correct for bulk refractive index changes and signal drift.

AFM

The AFM (East Coast Scientific (ECS) Cambridge, UK) was used with contact mode scanning, whereby the feedback loop maintains a constant force between the tip and surface. Samples may be imaged in air or under liquid. The AFM uses an optical detection system to measure cantilever deflection in which a laser is reflected off the free end of the cantilever onto a quadrant photodiode. AFM topographic images were acquired in contact mode under triethanolamine buffer, 0.1 M, pH 7 using non-oxide sharpened silicon nitride tips (Digital Instruments, Cambridge, UK).

Reflection absorption infrared spectroscopy (RAIRS)

Polarization-modulated RAIRS spectra were acquired on a Mattson Galaxy 4326 FTIR spectrometer upgraded with a photoelastic modulator (Hinds Instruments, Hillsboro, OR). The instrument was purged with nitrogen for at least 2 h before spectra were obtained. All spectra were obtained by recording at least 4000 scans at 4 cm^{-1} resolution.

Contact angle measurements

Sessile drop contact angle measurements were recorded using a CCD camera attached to a computer. Water drops (2 μl) were generated with a microsyringe, and at least 15 measurements on a number of different samples were taken for each monolayer. Electronic images of sessile drops were captured and analyzed using National Institutes of Health Image software.

RESULTS AND DISCUSSION

This paper describes the series of experiments that were involved in characterizing the immobilization of an enzyme on the tip of an AFM, and then measuring the interactions between the enzyme and two active site ligands, one in solution (ATP) and one (adenine) immobilized on a surface. The additional value of this work is that it shows the careful control experiments that are necessary to establish the integrity of the immobilized enzyme, using combinations of AFM, SPR, and kinetic measurements, and to characterize the complementary surface presenting the adenine substrate mimic. Shikimate kinase was chosen as the enzyme target because like many pharmaceutical targets, relatively little is known about its kinetic mechanism and inhibition; however, it is a prototype for the important classes of ATP-dependent enzymes, most notably the protein kinases.

The adhesion experiments suggest that the interaction between the immobilized enzyme and the ATP mimic were specific. The adhesion force was only reduced in the presence of ATP. The reduction was linear with ATP concentration up to the maximum concentration used. The addition of shikimic acid had no effect. It is known from the crystal structure that there is no significant overlap between the two substrate sites. As a further control the methyl ester of shikimic acid was used. This compound would not be expected to bind, and did not. The experiments with magnesium phosphate and the concentration of background electrolyte rule out an interpretation via non-specific electrostatic interactions. Although the nature of the interactions between shikimate kinase and the adenine alkanethiol SAM are not fully understood, the adhesion data obtained under increasing buffer concentrations is consistent with the interaction being a combination of specific binding interactions and a less specific hydrophobic interaction. It is known that the active site for shikimate kinase is a hydrophobic cleft, and molecular dynamic simulations on other kinase enzymes, e.g., CDK2, have found that the exclusion of water is the primary driving force for the interaction of ATP (Schulze-Gahmen et al., 1996). Literature examples suggest that hydrophobic interactions can act over surprisingly large distances; in some cases, distances up to 100 nm have been reported (Israelachvili, 1992).

The measurement of enzyme ligand interactions is of central importance to drug discovery, and several assay systems have been developed to facilitate the measurement of this interaction. The demonstration that chemical force microscopy can measure specific and competitive interactions between an enzyme and two active site ligands, one in solution and one immobilized on a surface, opens up the way for studies to identify novel ligands for other kinases, by screening compounds in solution either individually or in mixtures. Immobilization of other known enzyme inhibitors likewise will allow other classes of enzymes to be tested against potential inhibitors in solution. This approach has potential advantages over SPR studies of the binding small molecules to immobilized enzymes, where the small change in mass can be difficult to detect, albeit not in our studies on shikimate kinase (above). It also is a more practical approach to screening libraries of compounds by AFM than, for example, rastering the AFM tip over spatially arrayed libraries of small molecules (McKendry et al., 1999). The alternative strategy that is being explored vigorously at the present time is to use the AFM to study single unbinding events in complex proteins. There can be no doubt that this approach yields the ultimate in sensitivity and biophysical

detail. However, there remains great practical value in gaining accurate binding energies of enzyme inhibitors, and in this context it is valuable to use blunt tips to obtain an ensemble measurement from a small number of force-distance curves.

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