Selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands

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This paper describes a method for the selective and covalent immobilization of proteins to surfaces with control over the density and orientation of the protein. The strategy is based on binding of the serine esterase cutinase to a self-assembled monolayer presenting a phosphonate ligand and the subsequent displacement reaction that covalently binds the ligand to the enzyme active site. Surface plasmon resonance (SPR) spectroscopy showed that cutinase binds irreversibly to a monolayer presenting the capture ligand at a density of 1% mixed among tri(ethylene glycol) groups. The covalent immobilization is specific for cutinase, and the glycol-terminated monolayer effectively prevents unwanted nonspecific adsorption of proteins. To demonstrate that the method could be used to immobilize proteins of interest, a cutinase-calmodulin fusion protein was constructed and immobilized to the monolayer. SPR showed that calcineurin selectively associated with the immobilized calmodulin. This capture ligand immobilization method combines the advantages that the immobilization reaction is highly selective for the intended protein, the tether is covalent and, hence, stable, and the method avoids the need for synthetic modification and rigorous purification of proteins before immobilization. These characteristics make the method well suited to a range of applications and, in particular, for constructing protein microarrays.

Many experimental approaches in biology and applications in diagnostics and drug discovery require proteins immobilized on substrates (1–3). Of particular relevance is the recent introduction of protein microarrays—prepared by immobilizing hundreds or thousands of different proteins to a common substrate—that allow highly parallel experiments with small amounts of proteins and reagents. MacBeath and Schreiber (4), for example, immobilized a series of proteins on aldehyde-terminated glass slides and showed that these proteins were able to interact with other molecules in solution. More recently, Snyder and coworkers (5) immobilized a library of oligohistidine fusion proteins from *Saccharomyces cerevisiae* onto Ni-nitrilotriacetic acid (NTA) surfaces and used the array to identify several calmodulin- and phospholipid-binding motifs. These examples demonstrate the potential of protein arrays, but have also highlighted the need for methods that present proteins in a well defined environment and simultaneously prevent unwanted nonspecific protein interactions. This paper presents a method for the selective and irreversible immobilization of proteins to self-assembled monolayers of alkanethiolates on gold by using active site-directed ligands that first bind to a specific protein and subsequently react with the protein to give a covalent link.

A variety of methods are commonly used for immobilizing proteins. The most widely used method relies on nonspecific adsorption of the protein to a solid support (6). Simple chemical couplings of reactive groups within proteins (amines, acids) also have been used to immobilize proteins to surfaces comprising complementary reactive groups (4). Both methods, which require highly purified proteins, often result in randomly oriented and partially denatured proteins. The use of recombinant tags allows proteins to attach to a substrate in a defined orientation, but the interactions of the tags are reversible (e.g., glutathione *S*-transferase, oligohistidine) and, hence, are not stable over the course of subsequent assays or require large mediator proteins (e.g., biotin-streptavidin, antigen-antibody; refs. 7–9). A further disadvantage with all of these methods is that they are not well suited to controlling the densities of immobilized proteins. For these reasons, we sought a general method that would selectively immobilize proteins with absolute control over orientation and density and that does not require synthetic modification or purification before immobilization.

New methods for protein immobilization should have several characteristics to make them well suited to the preparation of protein arrays. First, the substrates that are used for immobilization must be inert to the nonspecific adsorption of protein. Inert substrates are necessary to prevent denaturation of immobilized proteins and unwanted adsorption of proteins in assays (which leads to both false positive and negative responses in assays and large background signals that limit sensitivity). Second, the reactions that covalently link the protein to the substrates should be selective, so the need to rigorously purify the protein is avoided and the protein is immobilized in a defined orientation. Third, the reaction should be rapid so that only small amounts of protein are necessary, and that problems associated with solvent evaporation are minimized. Fourth, the method should provide control over the density of immobilized protein, to ease steric interactions of neighboring proteins and to ensure that the amount of immobilized protein is reproducible. Finally, the resulting substrate should be compatible with several of the important detection technologies used in reading protein microarrays, including phosphorimaging, fluorescence, surface plasmon resonance (SPR) and mass spectrometry.

Our method is based on the active site-directed covalent immobilization of fusion proteins to surfaces presenting capture ligands (Fig. 1*A*). The fusion comprises a capture protein—in this work we used cutinase—and the protein of interest to be displayed at the surface. Cutinase is a 22-kDa serine esterase that forms a site-specific covalent adduct with phosphonate ligands (10). The phosphonate, which mimics the tetrahedral transition state of an ester hydrolysis, is attacked by the catalytic serine (Ser-120) residue, resulting in displacement of the leaving group and formation of a stable covalent adduct that is resistant to hydrolysis.

A crystal structure of the enzyme bound to the inhibitor reveals several characteristics that make this system well suited for use as an immobilization strategy (Fig. 1*B*; ref. 11). The alkyl group of the inhibitor protrudes from the active site into the

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Abbreviations: NTA, nitrilotriacetic acid; SPR, surface plasmon resonance; Cut-CaM, cutinase-calmodulin; EST, expressed sequence tag.

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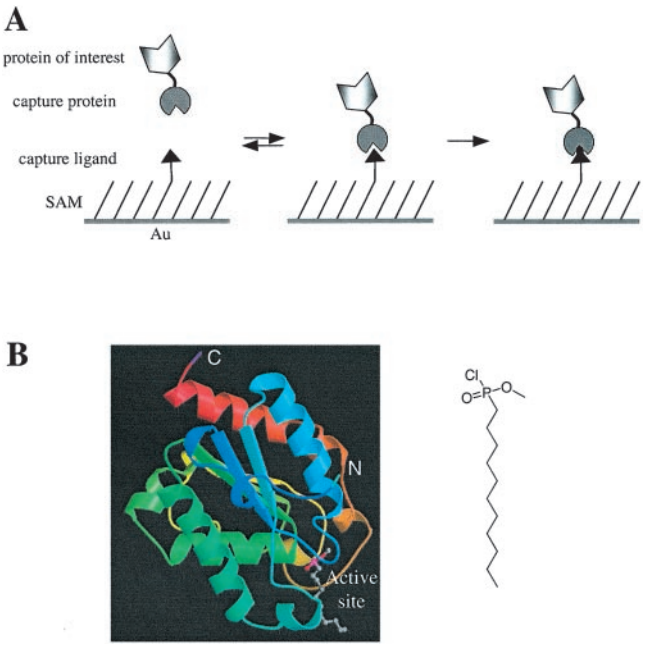


Fig. 1. (*A*) Strategy for protein immobilization. The protein of interest is fused to a capture protein, which specifically binds a capture ligand of the substrate to give covalent immobilization of proteins while maintaining activity and orientation. The density of immobilized protein can be controlled by adjusting the density of the ligand. (*B*) Structure of *F. solani* cutinase bound to an *n*-undecyl *o*-methyl chlorophosphonate ester inhibitor (11). The inhibitor is covalently bound through an active-site serine residue.

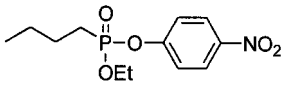
solvent, which provides an accessible point for immobilization to a surface. The enzyme is small, globular, and monomeric; these characteristics minimize steric effects with the proteins to be immobilized. The termini are opposing the active site, so that either N- or C-terminal fusions can be formed and still orient the display protein away from the substrate. Although this attachment controls the orientation of the display protein, conformational flexibility in the tether between the two domains can lead to a small range of orientations. This system has the additional advantages that the inhibitor is synthesized in a straightforward manner and is relatively stable in water. Below, we describe experiments that use SPR spectroscopy to characterize the immobilization of cutinase to monolayers presenting the capture ligand. Then, we demonstrate the use of this strategy to immobilize the protein calmodulin and show that surfaces presenting this protein are active and specifically bind the protein calcineurin.

Materials and Methods

General. *Fusarium solani pisi* (#38120) was purchased from American Type Culture Collection, and the expressed sequence tag (EST) clone encoding human calmodulin (IMAGE, 2629459) was obtained from ResGen. Calcineurin was obtained from Sigma, oligonucleotides were from Genosys, and enzymes were from New England Biolabs or Promega. Spectrophotometric measurements were performed at room temperature with a Beckman DU-640 spectrophotometer. [^{1}H] NMR spectra were recorded on a Bruker 500 MHz spectrometer in $CDCl_3$, with chemical shifts reported relative to the residual solvent peak (for spectra, see supporting information, which is published on the PNAS web site, www.pnas.org). [^{31}P] NMR was recorded on a Bruker 500 MHz spectrometer in $CDCl_3$ with chemical shifts reported relative to H_3PO_4. Reagents were used as received unless otherwise stated. Solvents were dried by using standard procedures. Chromatography was carried out by using Merck Silica gel 60 (230–400) mesh. TLC was performed on Whatman silica gel plates (0.25 mm thickness).

Construction of Plasmids. Plasmids were maintained and propagated in *Escherichia coli* (DH5α, Novagen). The cutinase gene containing two exons and an intron was amplified from *F. solani* genomic DNA by using the following primers: 5′-GCCACGGCCATGGGCCTGCCTACTTCTAACCCTGCCCAGGAG-3′ and 5′-CCGGGATCCTCAAGCAGAACCACGGACAGCCCGAAC-3′. The exons then were amplified separately by using the following additional primers: 5′-CCGGTACCCAAGTTGCCCGTCTCTGTTGAACCTCGGGC-3′ and 5′-CCGGTACCCTCGGTCCTAGCATTGCCTCCAACCTTGAG-3′. The two exons were digested with *Kpn*I, ligated with T4 DNA ligase, and the correct ligation product (cutinase gene) was purified by agarose gel (1.5%) electrophoresis. The DNA then was digested with *Nco*I and *Bam*HI and then cloned into pET-22b(+) (Novagen). The resulting plasmid (pCut22b) encodes a gene for cutinase whose N-terminal leader sequence was replaced with a pelB leader sequence, which allows for periplasmic localization of the expressed protein. To construct the plasmid encoding cutinase-fusion proteins, the cutinase gene without the stop codon was amplified by using PCR and ligated into pET-22b(+) via *NcoI* and *BamHI* sites, resulting in pCut22b(−Z). The DNA coding for CaM was amplified from an EST by using the following primers: 5′-CCGGGATCCGCTGATCAGCTGACCGAAGAA-3′ and 5′-AGGATCCTCGAGTTTTGCAGTCATCATCTGTAC-3′. The PCR product was cloned into pCut22b(-Z) via *Bam*HI and *Xho*I sites, resulting in pCutCaM. The presence of the correct inserts was confirmed by bidirectional DNA sequencing.

Expression and Purification of Proteins. Cutinase and Cut-CaM were expressed in *E. coli* [BL21(DE3), Novagen] harboring pCut22b and pCutCaM, respectively, with a T7 expression system. Cells were grown at 37°C to A_{600} = 0.3 before induction with 0.2 mM isopropyl β-D-thiogalactoside at 25°C for 12 h. Cells were collected by centrifugation at 5,000 × *g* for 30 min and then subjected to sucrose osmotic shock to release the periplasmic proteins (12). SDS/15% PAGE showed the protein was 80% pure with the major band corresponding to a molecular mass of 22 kDa, which is the expected size of cutinase. Cutinase was purified by size-exclusion chromatography by using a Sephadex G-75 column (75 cm × 2 cm, 1 ml/min) equilibrated in PBS, pH 7.4 at 4°C to afford 20 mg of protein from a 1 liter culture. We further characterized cutinase by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (m/z_{exp} = 22,515.89; m/z_{calc} = 22,421). Protein was concentrated by using a Centriprep YM-10 (Millipore), and concentrations were determined with a calculated extinction coefficient (ε_{280} = 13,370 $M^{-1}\cdot cm^{-1}$) in denaturing conditions (10 mM sodium phosphate, pH 6.5/6.0 M guanidine·HCl). Enzyme activity was confirmed by monitoring the hydrolysis of *p*-nitrophenyl butyrate as described (13). Periplasmic fractions containing Cut-CaM were loaded onto 10-ml Ni-NTA agarose columns (Sigma) equilibrated in buffer (PBS, pH 7.4 and 20 mM imidazole). The column was washed with five volumes of the same buffer, and then bound proteins were eluted with 200 mM imidazole in PBS. Fractions containing Cut-CaM were identified by SDS/12% PAGE and dialyzed against 3 × 1 liter PBS (pH 7.4) at 4°C to afford 5 mg of protein from a 1 liter culture at greater than 95% purity. Protein concentrations were determined by using a calculated extinction coefficient (ε_{280} = 15,930 $M^{-1}\cdot cm^{-1}$), as described above.



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Scheme 1.

Assay for Inhibition. Solutions of inhibitor **1** (inhibitor dissolved in 100 μl of DMSO) were added to identical solutions of cutinase in PBS [900 μl, pH 7.4; 0.04% (vol/vol) Triton X-100] to give final inhibitor concentrations ranging from 3 to 100 μM and a final cutinase concentration of 25 μM. The time-dependent release of *p*-nitrophenol was measured with a UV-vis spectrophotometer at 405 nm (14). The initial rates of inactivation were plotted against the initial inhibitor concentrations and analyzed by standard methods to obtain kinetic parameters. As a control, a solution of inactivated cutinase was dialyzed against PBS (pH 7.4) for 24 h and found to have no esterase activity.

SPR Spectroscopy. Substrates for SPR were prepared as described (15). Monolayers were formed by immersion of substrates in an ethanolic solution containing disulfide **6** (Fig. 2 **6**) and the symmetrical disulfide 2-(2-{2-[11-(11-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-undecyldisulfanyl)-undecyloxy]-ethoxy}-ethoxy)-ethanol for 12 h (ratio of 1:99; 1.0 mM total concentration) as described (16). SPR experiments were performed with a Biacore 1000 instrument. All experiments used a flow rate of 1 μl/min at 25°C in PBS (pH 7.4) containing various additives, as described in the text.

Synthesis of Imidazole-1-carboxylic Acid 11-(Diethoxy-phosphoryl)-undecyl Ester (3). To a solution of alcohol **2** (see Fig. 2, 485 mg, 1.57 mmol) dissolved in 10 ml of CH_2Cl_2 was added freshly sublimed 1,1′-carbonyldiimidazole (510 mg, 3.15 mmol; ref. 17). After stirring at room temperature for 10 h, the reaction mixture was rinsed with H_2O (2 $\times$ 10 ml). The organic layer was dried over $MgSO_4$ and concentrated to give 507 mg (80%) of pure **3** (see Fig. 2 **3**) as a white solid. [^{1}H] NMR ($CDCl_3$, 500 MHz) δ 8.22 (s, 1H), 7.44 (s, 1H), 7.10 (s, 1H), 4.41 (t, J = 6.5 Hz, 2H), 4.07 (m, 4H), 1.77 (m, 2H), 1.69 (m, 2H), 1.57 (m, 2H), 1.44–1.21 (br m, 20H). [^{31}P] NMR ($CDCl_3$, 500 MHz) δ 8.22.

Synthesis of Imidazole-1-carboxylic Acid 11-[Ethoxy-(4-nitrophenoxy)-phosphoryl]-undecyl Ester (4). To a solution of **3** (1.2 g, 3.0 mmol) dissolved in 25 ml of CH_2Cl_2 was added oxalyl chloride (0.65 ml, 7.5 mmol) dropwise at 0°C. The reaction mixture was allowed to slowly warm to room temperature. After stirring for 8 h, the mixture was concentrated to remove excess oxalyl chloride. The crude residue was redissolved in 20 ml of CH_2Cl_2, followed by the addition of 4-nitrophenol (414 mg, 3.0 mmol) and Et_3N (0.80 ml, 6.0 mmol). After stirring at room temperature for 10 h, the reaction mixture was concentrated. The residue was purified by flash chromatography (hexanes:EtOAc, 1:1) to give 601 mg (41%) of pure **4** (see Fig. 2 **4**) (1).H NMR ($CDCl_3$, 500 MHz) δ 8.16 (d, J = 9.0 Hz, 2H), 8.10 (s, 1H), 7.37 (s, 1H), 7.32 (d, J = 9.0 Hz, 2H), 7.01 (s, 1H), 4.35 (t, J = 6.5 Hz, 2H), 4.11 (m, 2H), 1.87 (m, 2H), 1.72 (m, 2H), 1.62 (m, 2H), 1.38–1.17 (br m, 20H). [^{31}P] NMR ($CDCl_3$, 500 MHz) δ 30.49.

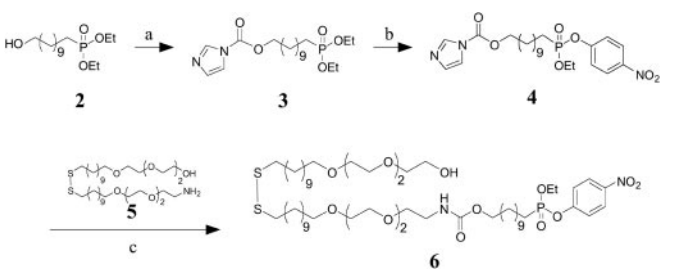

Fig. 2. Synthetic scheme for the phosphonate disulfide (**6**) used to prepare self-assembled monolayers. (a) CDI, CH_2Cl_2; (b) oxalyl chloride, CH_2Cl_2; 4-nitrophenol, Et_3N, CH_2Cl_2; (c) **5**, Et_3N, DMF.

Synthesis of {11-[2-(2-{2-[11-(11-{2-[2-(2-Hydroxy-ethoxy)-ethoxy]-ethoxy}-undesyldisulfanyl)-undecyloxy]-ethoxy}-ethoxy)-ethylcarbamoyloxy]-undecyl}-phosphonic Acid Ethyl Ester 4-Nitro-phenyl Ester (6). A solution of phosphonate **4** (68 mg, 0.14 mmol), amino disulfide **5** (see Fig. 2 **5**; 120 mg, 0.25 mmol), and Et_3N (35 μl, 0.25 mmol) dissolved in dimethylformamide (DMF) was stirred at 50°C for 60 h (18). After concentration of the reaction mixture, the crude residue was purified by column chromatography (CH_2Cl_2:MeOH, 20:1) to afford 18 mg (20%) of pure disulfide **6**. [^{1}H] NMR ($CDCl_3$, 500 MHz) δ 8.22 (d, J = 9 Hz, 2H), 7.37 (d, J = 9.0 Hz, 2H), 4.30–4.10 (m, 2H), 3.98 (m, 2H), 3.70–3.20 (m, 30H), 2.66 (t, J = 7.5 Hz, 4H), 1.90 (m, 2H), 1.80–1.60 (m, 8H), 1.60–1.00 (m, 47H).

Results and Discussion

Cloning and Expression of Cutinase. The cutinase gene, which comprises two exons separated by a 50-bp intron, was isolated from the fungus *F. solani*. Exon 1 and exon 2 were amplified with primers that introduced the restriction sites, *Nco*I and *Kpn*I and *Kpn*I and *Bam*HI on the 5′ and 3′ ends, respectively. The two exons then were ligated by their common *Kpn*I site to yield the cutinase cDNA (630 bp) which was purified from other ligation products by agarose gel (1.5%) electrophoresis. To facilitate the proper folding of cutinase, which contains two disulfide bridges, we replaced the original leader sequence of cutinase with a pelB leader sequence and, thus, allowed for its transport to the nonreducing environment of the periplasm of *E. coli*. After cloning into pET-22b(+), cutinase was expressed in *E. coli* to afford high levels of active enzyme.

Irreversible Inhibition of Cutinase by Phosphonate 1. We chose the 4-nitrophenyl phosphonate inhibitor because it is more stable toward hydrolysis than the chlorophosphonates (as shown in Fig. 1*B*), and its reaction with cutinase can be measured by absorbance spectroscopy. We first characterized the irreversible inhibition of cutinase by 4-nitrophenyl phosphonate **1** (14). Inhibitor **1** at concentrations ranging from 3 to 100 μM were added to identical solutions of cutinase (12 μM), and the rate of release of 4-nitrophenol was followed by absorbance spectroscopy. Standard treatment of the rate of inactivation for each concentration of inhibitor provided both an equilibrium binding constant between inhibitor and protein (K_i = 65.5 μM) and a first-order rate constant for reaction of the bound complex (k_{inact} = 0.02 s^{-1}; ref. 19). When the inhibited enzyme was incubated in the solution containing 4-nitrophenyl butyrate, no release of 4-nitrophenolate was observed, showing that the inhibited enzyme does not possess esterase activity.

Preparation of SAMs for Protein Immobilization. To incorporate the phosphonate capture ligand into SAMs, we synthesized phosphonate dialkyldisulfide **6**. The activated imidazole carbamate **3** was prepared from the previously described diethyl phosphonate **2** by reaction with 1,1′-carbonyldiimidazole (17). Chlorination of **3** with oxalyl chloride followed by substitution with 4-nitrophenol gave the 4-nitrophenyl-activated phosphonate **4**. Amino disulfide **5**, which was prepared as previously described, was coupled with intermediate **4** to afford disulfide **6** (18). SAMs presenting the phosphonate-terminated capture ligand were prepared by immersing gold-coated glass in solutions containing disulfide **6** with a symmetrical EG_3 disulfide (Fig. 3; ref. 16). The density of

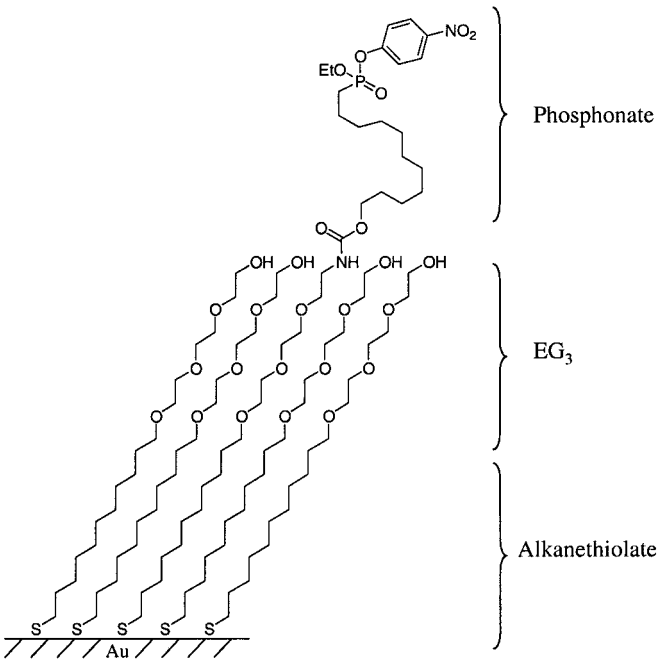

Fig. 3. Structure of a monolayer designed to covalently immobilize cutinase. The phosphonate capture ligand is present at a density of ≈1% of total alkanethiolate. After cutinase binds to the phosphonate capture ligand, an active-site serine residue displaces *p*-nitrophenol, resulting in irreversible covalent immobilization of the protein. The tri(ethylene glycol) groups prevent nonspecific protein adsorption to the monolayer.

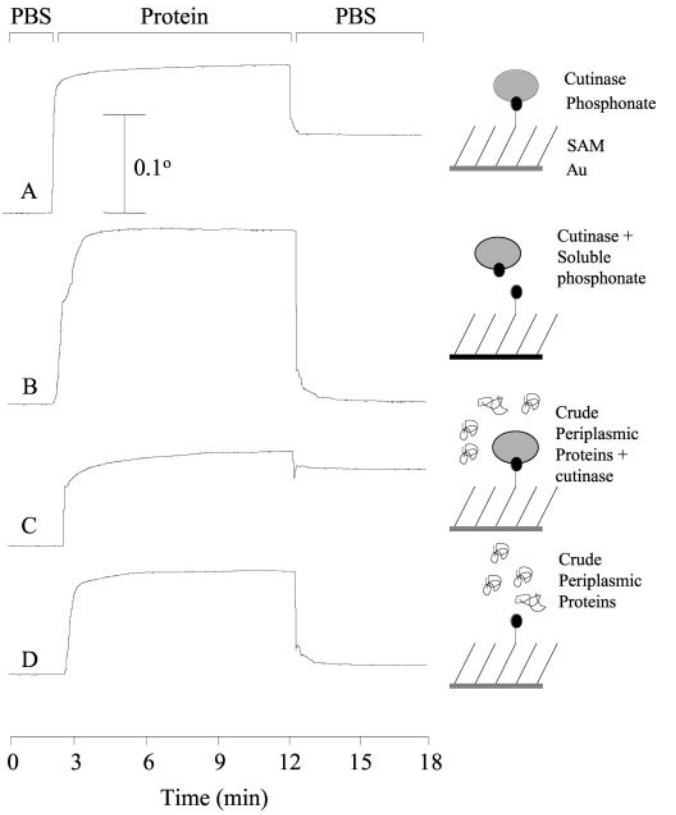

Fig. 4. SPR spectroscopy data showing the biospecific and irreversible immobilization of cutinase to the phosphonate-tethered SAM shown in Fig. 3. Conditions are described in *Materials and Methods*. The change in resonance angle ($\Delta\theta$) is plotted on the vertical axis. The scale bar applies to all data, which are offset for clarity. (*A*) Cutinase (25 μM) was irreversibly immobilized to the monolayer. (*B*) Incubation of cutinase with four equivalents of soluble inhibitor **1** before immobilization completely inhibited immobilization of the protein. (*C*) Cutinase within crude *E. coli* periplasmic lysate also underwent efficient immobilization, demonstrating that the protein does not need to be purified before immobilization. (*D*) An analogous experiment using periplasmic lysate without cutinase resulted in no immobilization and demonstrates that the monolayer is resistant to nonspecific protein adsorption.

the capture ligand—which can be adjusted by varying the ratio of the two disulfides—was ≈1% mixed with tri(ethylene glycol) groups which resist nonspecific protein adsorption.

Immobilization of Cutinase to SAM. We used SPR spectroscopy to characterize the immobilization of cutinase to SAMs. In these experiments, PBS (pH 7.4) was flowed over the monolayer for 2 min to establish a baseline, followed by a solution of protein in the same buffer for 10 min to observe binding. Finally, the protein solution was replaced with buffer for 6 min to quantitate the amount of protein that was irreversibly immobilized. Cutinase (25 μM) bound irreversibly to the monolayer (Fig. 4*A*). Treatment of the monolayer with SDS (0.5 mg/ml) did not result in removal of cutinase from the surface (data not shown), supporting the result that the immobilization was covalent (SDS is a detergent that serves to remove noncovalently immobilized molecules from a surface; ref. 20). The immobilization was prevented when cutinase was first blocked with four equivalents of **1** (Fig. 4*B*), demonstrating that the immobilization was biospecific. We next tested the specific immobilization of cutinase from crude *E. coli* periplasmic extracts (total protein concentration = 0.7 mg/ml) and found that the same amount of binding was observed as in the case of purified cutinase (Fig. 4*C*); again, the protein was not removed on rinsing with SDS. Periplasmic lysate of *E. coli* that was not transformed with the cutinase plasmid gave no immobilization to the monolayer (Fig. 4*D*), providing additional evidence that even from crude extracts, the immobilization of cutinase is specific and the monolayer remains inert to the nonspecific adsorption of protein.

Immobilization and Activity of Cutinase-Calmodulin Fusion. To validate that this strategy could be applied to the immobilization of other proteins, we constructed a Cut-CaM fusion protein. We chose calmodulin because it selectively binds the protein calcineurin in the presence of calcium ion, with a dissociation constant of 20 nM (21). SPR showed that the fusion protein immobilized to a SAM presenting the phosphonate inhibitor (Fig. 5*A*), and that the immobilized protein was again stable to SDS (data not shown). Calcineurin (CaN) was allowed to interact with the calmodulin-presenting surface constructed as in Fig. 5*A* in the presence of Ca^{2+} (Fig. 5*B*). Calcineurin bound to the substrate, and several control experiments established that the binding was specific. First, binding of calcineurin to the immobilized CaM was completely inhibited by the addition of EGTA (2 mM), which chelates the calcium ions required for recognition (Fig. 5*C*). Second, the addition of soluble calmodulin to the calcineurin-containing solution also inhibited association with the immobilized CaM (Fig. 5*D*). Finally, calcineurin did not bind to a surface to which only cutinase was immobilized (Fig. 5*E*). These data clearly show that the interaction between calcineurin and the surface was mediated by the immobilized calmodulin and, therefore, that the cutinase fusion strategy can be applied to immobilizing other proteins.

This example establishes a new strategy for protein immobilization that shares the advantages that binding of capture protein to the immobilized capture ligand is selective, reaction of the capture ligand with the active site gives a covalent

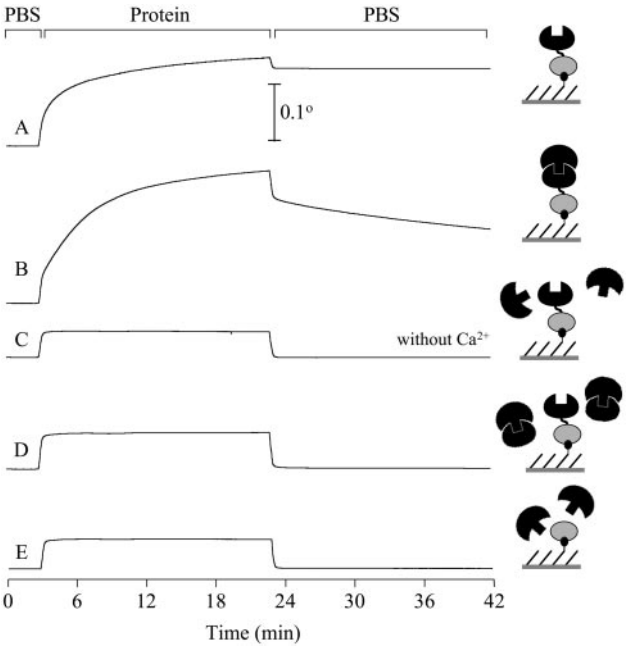

Fig. 5. SPR data for binding of calcineurin to surfaces presenting calmodulin (CaM). (*A*) The Cut-CaM fusion (1 μM) irreversibly immobilized to SAMs presenting the phosphonate capture ligand. (*B*) Calcineurin (0.7 μM, 0.2 mM $CaCl_2$) binds to immobilized CaM in the presence of Ca^{2+} ions. (*C*) Calcineurin is unable to bind CaM in the presence of the divalent ion chelator EGTA (2 mM), demonstrating the known requirement of Ca^{2+} for binding. (*D*) Incubation of calcineurin with soluble CaM (2 μM) also prevents binding of calcineurin, showing that the interaction between immobilized CaM and calcineurin is biospecific. (*E*) calcineurin does not bind to a surface presenting cutinase alone, demonstrating that calcineurin binding is not mediated by cutinase.

immobilization, and the recombinant expression of the capture protein avoids the need for synthetic modification and purification of proteins. The successful development of this mechanism-based approach to immobilization relied on two important developments in surface chemistry. First, the structural order of self-assembled monolayers of alkanethiolates on gold together with the ability to prepare monolayers from highly functionalized alkanethiols provides an unprecedented opportunity to engineer surfaces with designated properties (22). Second, the use of monolayers presenting oligo(ethylene glycol) groups prevents unwanted nonspecific adsorption of protein and is critical to realizing specific interactions between the capture proteins and the substrate. We note that this strategy should prove general for the use of other pairs of capture protein and capture ligand and thereby produce several new methods for protein immobilization.

We believe the capture ligand strategy will prove important for a range of applications. We have emphasized protein chips as an important application because the requirement to immobilize hundreds or thousands of different proteins presents a difficult challenge to existing immobilization methods. Another important application is the development of substrates that serve as models of extracellular matrix for studies of cell adhesion and migration. Because cell adhesion and signaling pathways strongly depend on the density of immobilized ligands, it is important that the densities of immobilized proteins be controlled (23). Finally, the capture ligand strategy may prove important for efforts in materials science that incorporate protein assemblies as functional components (24).

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1. Fields, S. (2001) *Science* **291,** 1221–1224.
2. Zhu, H. & Snyder, M. (2001) *Curr. Opin. Chem. Biol.* **5,** 40–45.
3. MacBeath, G. (2001) *Nat. Biotechnol.* **19,** 828–829.
4. MacBeath, G. & Schreiber, S. L. (2000) *Science* **289,** 1760–1763.
5. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayer, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T. (2001) *Science* **293,** 2101–2105.
6. Arenkov, P., Kukhtin, A., Gemmell, A., Voloshchuk, S., Chupeeva, V. & Mirzabekov, A. (2000) *Anal. Biochem.* **278,** 123–131.
7. Saleemuddin, M. (1999) *Adv. Biochem. Eng. Biotechnol.* **64,** 203–226.
8. Turkova, J. (1999) *J. Chromatogr. B* **722,** 11–31.
9. Holt, L. J., Enever, C., de Wildt, R. M. & Tomlinson, I. M. (2000) *Curr. Opin. Biotechnol.* **11,** 445–449.
10. Mannesse, M. L., Boots, J. W., Dijkman, R., Slotboom, A. J., van der Hijden, H. T., Egmond, M. R., Verheij, H. M. & de Haas, G. H. (1995) *Biochim. Biophys. Acta* **1259,** 56–64.
11. Longhi, S., Nicolas, A., Creveld, L., Egmond, M., Verrips, C. T., de Vlieg, J., Martinez, C. & Cambillau, C. (1996) *Proteins* **26,** 442–458.
12. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989) *Current Protocol in Molecular Biology* (Wiley, New York).
13. Kolattukudy, P. E., Purdy, R. E. & Maiti, I. B. (1981) *Method. Enzymol.* **71,** 652–664.
14. Wu, S.-Y. & Casida, J. E. (1995) *Chem. Res. Toxicol.* **8,** 1070–1075.
15. Mrksich, M., Sigal, G. S. & Whitesides, G. M. (1995) *Langmuir* **11,** 4383–4385.
16. Hodneland, C. D. & Mrksich, M. (2000) *J. Am. Chem. Soc.* **122,** 4235–4236.
17. Deussen, H. J., Danielsen, S., Breinholt, J. & Borchert, V. T. (2000) *Bioorg. Med. Chem.* **8,** 507–514.
18. Hodneland, C. D. (2001) Ph.D. thesis (Univ. of Chicago).
19. Björkling, F., Dahl, A., Patkar, S. & Zundel, M. (1994) *Bioorg. Med. Chem.* **2,** 697–705.
20. Sigal, G. S., Mrksich, M. & Whitesides G. M. (1998) *J. Am. Chem. Soc.* **120,** 3464–3473.
21. Takano, E., Hatanaka, M. & Maki, M. (1994) *FEBS Lett.* **352,** 247–250.
22. Houseman, B. T., Huh, J. H., Kron, S. J. & Mrksich, M. (2002) *Nat. Biotechnol.* **20,** 270–274.
23. Houseman, B. T. & Mrksich, M. (2001) *Biomaterials* **22,** 943–955.
24. Lee, I., Stubna, A. & Greenbaum, E. (2000) *J. Phys. Chem. B* **104,** 2439–2446.