# Two-Step Affinity Chromatography. Model Systems and an Example Using Biotin–Avidin Binding and a Fluoridolyzable Linker[1]

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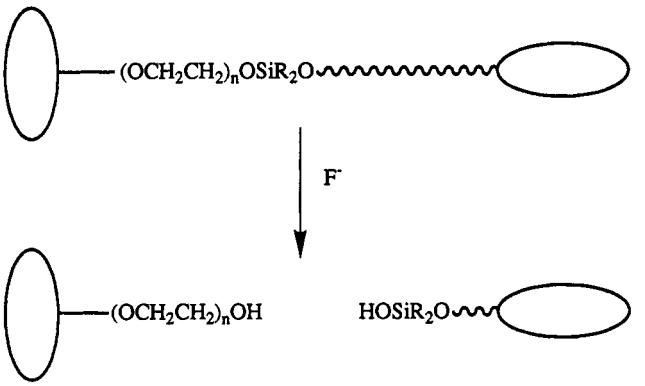
Two-step affinity chromatography is a general designation for a separation procedure that involves the following pair of sequential steps: specific binding of a biomolecule to a solid support then selective elution using a chemically specific cleaving agent to sever the tether that is attached to the biomolecule. This procedure is exemplified for the case of the enzyme papain covalently modified at its active site. A molecule of biotin is connected to a cysteine residue via a hydrophilic tether incorporating a fluoridolyzable $-CH_2OSiMe_2OCMe_2-$ unit. The biotinylated papain then binds a molecule of streptavidin (a protein that has four identical binding sites for biotin), and this complex in turn binds to a molecule of biotin that is covalently linked to a solid support. After this first step, the covalently modified papain is then selectively eluted by fluoride-catalyzed hydrolysis of an oxygen–silicon bond in the hydrophilic tether. Homogeneous kinetics of model systems in aqueous solution show no evidence for saturation of the rate of fluoride-catalyzed hydrolysis of silicon–oxygen bonds.

Biology provides many examples of stable covalent bonds that readily hydrolyze under appropriate catalytic conditions. One objective of biomimetic chemistry is to prepare synthetic analogues, i.e., to construct bonds that are stable under physiological conditions, which can be cleaved at will. Ideally, the catalyst for cleavage ought to have a low physiological abundance and ought not to denature proteins. Fluoride ion fulfills these requirements. "Fluoridolysable" linkages have been described as useful intermediates in synthetic chemistry.[2] The function of such linkages, which use silicon-based groups, might be viewed as biomimetic, apart from the fact that they are only known to operate in nonaqueous systems. The question arises whether fluoridolyzable silicon linkages can be used in aqueous solution at physiological pH. We present here an affirmative answer.

Diaryldialkoxysilanes with hydrophilic extender arms (to render them water soluble) have been examined and are found to satisfy two requisites for use as reversible linkers in biological systems: (1) suitable stability in aqueous solution at physiological pH and (2) substantial acceleration of hydrolytic cleavage in the presence of fluoride. Several uses may be envisaged for such a linkage. For instance, it might be used to tether two macromolecules, as Scheme I depicts, in which the ellipses represent the tethered moieties. The two large pieces at the top are hitched together and cannot get further apart than the length of the tether. Each macromolecule experiences a high local concentration of its partner. With a tether 20-Å long, for example, the effective concentration of one end with respect to the other (neglecting excluded volume) is 0.05 M. Cleavage of the tether by addition of catalyst releases the two ends to bulk solution, where their concentrations are much lower.

This can be applied to affinity chromatography, which is a widely applied separation technique for isolating molecules of biological interest. One popular application involves tagging an antibody or a ligand with biotin and

**Scheme I**




then attaching it to a solid support to which the protein avidin has been linked. A general representation of this technique is depicted in Scheme II. Once the antibody or ligand has been attached to the solid support, it can then be used as a stationary phase for purification of the antigen (in the case of an antibody) or of the receptor for the ligand,[3,4] as drawn in step iii of Scheme II. The use of biotin and avidin to link to the solid support depends upon the high affinity of avidin or streptavidin ($K_d \approx 10^{-14}$–$10^{-15}$ M) for biotin, which is an enzyme cofactor that has a molecular weight of 244 Da. Avidin has four identical binding sites, so it can be bound to a biotinylated solid support, step i, prior to attachment of a second biotin unit, step ii.

Consider the following variation of this procedure. A biotin moiety is covalently attached directly to the biomolecule of interest. The biomolecule is then purified by attachment to a molecule of avidin that is coupled to a biotinylated solid support. Despite the simplicity of this notion, such an approach has seen far less widespread application. Three obstacles stand in the way of successful implementation. First, the extender arms that are commercially available for attaching biotin are comparatively short and are composed of hydrophobic units (such as hydrocarbon chains). Therefore, direct attachment of avidin to another macromolecule (particularly if the latter is membrane-bound) cannot be relied upon.

Second, biotin is a naturally occuring coenzyme that is catalytically active only when it is covalently attached to a protein via an amide linkage with a lysine residue.

(1) Taken from the Ph.D. thesis of W.-C. Lin, U. C. Riverside, 1990. Portions of this work were presented at the 1987 Pacific Conference on Chemistry and Spectroscopy, Irvine, CA and at the 196th National Meeting of the American Chemical Society, Los Angeles, CA.

(2) (a) Mullen, D. G.; Barany, G. *Tetrahedron Lett.* **1987**, *28*, 491–494. (b) Ramage, R.; Barron, C. A.; Bielecki, S.; Thomas, D. W. *Tetrahedron Lett.* **1987**, *28*, 4105–4108. (c) Shea, K. J.; Zandi, K. S.; Staab, A. J.; Carr, R. *Tetrahedron Lett.* **1990**, *31*, 5885–5888. (d) Craig, D.; Reader, J. C. *Tetrahedron Lett.* **1990**, *31*, 6585–6588. (e) Hutchinson, J. H.; Daynard, T. S.; Gillard, J. W. *Tetrahedron Lett.* **1991**, *32*, 573–576 and references cited therein.

(3) For a recent example, see: Jans, D. A.; Bergmann, L.; Peters, R.; Fahrenholz, F. *J. Biol. Chem.* **1990**, *265*, 14599–14605.

(4) Wilchek, M., Bayer, E. A., Eds. Avidin-Biotin Technology. (*Methods Enzymol. 184*) Academic Press: Boston, 1990.

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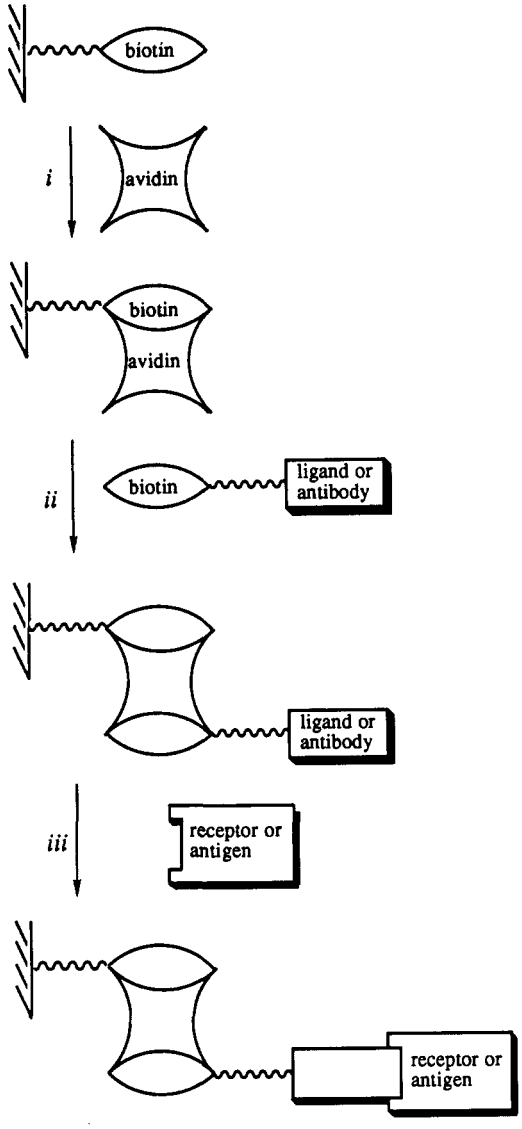
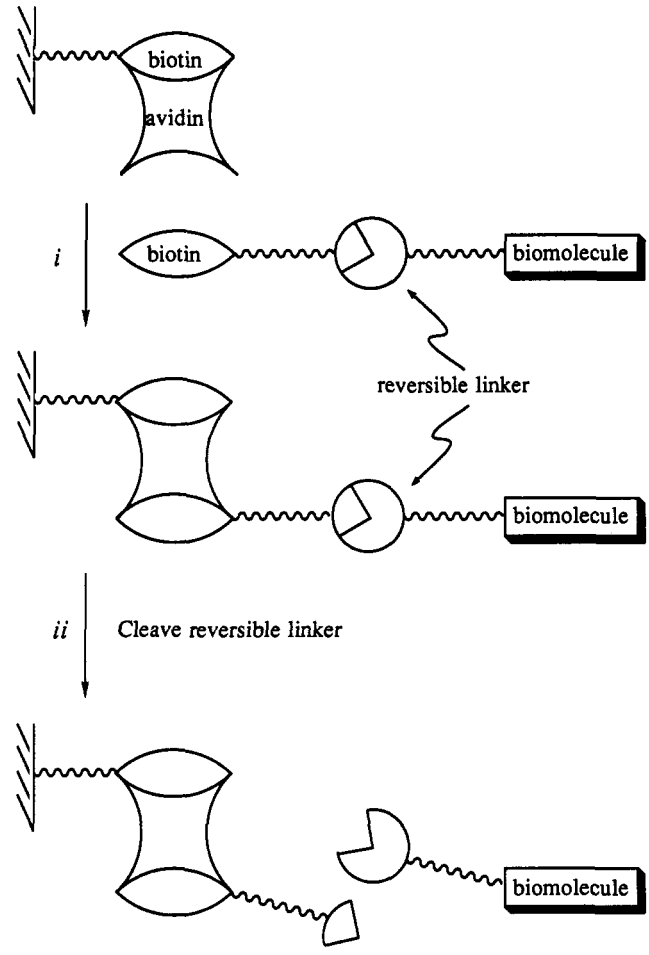
**Scheme II**




Therefore, most biological samples will contain several biotinylated proteins that can also bind to the affinity column. Third, the bound biomolecule must be released from the affinity column. When biotin is covalently attached to the biomolecule, either the biotin–avidin complex must be broken up (typically by denaturing the avidin, since flooding the column with excess free biotin will not readily elute the desired material, owing to the slow off rate of biotin) or else the tether must be severed. This latter strategy is illustrated in Scheme III. Candidates for a reversible linker that can be severed in the tether include disulfide linkages and *vic*-diols.[5] The conditions for cleaving these reversible linkers, however, are apt to modify the biomolecule if it is a sensitive protein. The second part of this paper presents the application of a fluoridolyzable linker, incorporated into a hydrophilic tether, to the problem of affinity chromatography of a modified protein. We call the general procedure represented by Scheme III "two-step affinity chromatography." Each step—specific binding and specific elution—provides a degree of purification. In particular, specific elution overcomes the obstacle of the natural background of biotinylated enzymes. This paper will describe the two-step affinity chromatographic purification of a covalently modified papain as an example of the technique.

**Scheme III**




## Experimental Section

**Materials.** Except as noted, reagents were obtained commercially and used without further purification. Pyridine, benzene, *tert*-butyl alcohol, triethylamine, 3-methyl-1,3-butanediol, triethyleneglycol monomethyl ether, and dichlorodimethylsilane were distilled from calcium hydride under nitrogen before use. Anhydrous DMF was obtained from Aldrich Chemical Co. and used without further treatment. Biotin-agarose was purchased from Pierce Chemical Co. Avidin, streptavidin, avidin-agarose, and streptavidin-agarose were purchased from Vector Laboratories, Incorporated, or Sigma Chemical Co. Papain was purchased from Boehringer Mannheim Co. and purified by affinity chromatography on a column of Gly-Gly-Tyr-Arg-agarose (the tetrapeptide was obtained from Chemical Dynamics Co. and attached to CNBr agarose).[6] Enzyme activity was determined spectrophotometrically by measuring the rate of hydrolysis of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPA).

**Diphenylbis[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]silane (2).** A solution of 4.9 g (30 mmol) of $H(OCH_2CH_2)_3OCH_3$ and 1.6 mL of pyridine in 10 mL of benzene was stirred under nitrogen at room temperature in a 50-mL round-bottom flask. A solution of 2.5 g (10 mmol) of diphenyldichlorosilane in 20 mL of benzene was added dropwise, and a white precipitate was observed. After 30 min the reaction mixture was filtered and the filtrate washed

(5) (a) Shimkus, M.; Levy, J.; Herman, T. *Proc. Natl. Acad. Sci. U. S.A.* **1985**, *82*, 2593–2597. (b) Shimkus, M.; Guaglianone, P.; Herman, T. *DNA* **1986**, *5*, 247–255; (c) Roseman, B.; Lough, J.; Houkom, E.; Herman, T. *Biochem. Biophys. Res. Commun.* **1986**, *137*, 474–479. (d) Rigas, B.; Welcher, A. A.; Ward, D. C.; Weissman, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9591–9595. (e) Chu, B. C. F.; Kramer, F. R.; Orgel, L. E. *Nucleic Acids Res.* **1986**, *14*, 5591–5603. (f) Marie, J.; Seyer, R.; Lombard, C.; Desarnaud, F.; Aumelas, A.; Jard, S.; Bonnafous, J.-C. *Biochemistry* **1990**, *29*, 8943–8950.

(6) Funk, M. O.; Nakagawa, Y.; Skochdopole, J.; Kaiser, E. T. *Int. J. Peptide Protein Res.* **1979**, *13*, 296–303.

with three 20-mL portions of water. Solvent was removed under reduced pressure and the residue purified by flash column chromatography using 30:70 (v/v) hexane/ethyl acetate. Phenyl-*tert*-butylbis[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]silane (**3**) was prepared in the same fashion from phenyl-*tert*-butyldichlorosilane. Mass spectrum of **2** (FAB, *m*-nitrobenzyl alcohol matrix): calcd for $C_{26}H_{41}O_8Si$ (M + H) 509.2571, found 509.2568.

**1-(Chloroacetoxy)-3-methyl-3-butanol (5).** Chloroacetyl chloride (12.1 g, 0.11 mol) in ether (200 mL) was added dropwise over a period of 6 h to a magnetically stirred solution of 3-methyl-1,3-butanediol (10.1 g, 0.97 mol) and pyridine (8.5 g, 0.11 mol) in ether (120 mL) at −15 °C under nitrogen, after which the reaction mixture was allowed to warm to room temperature. The mixture was then filtered to remove precipitated pyridinium hydrochloride and washed with saturated aqueous $CuSO_4$ (10 mL) and twice with saturated aqueous NaCl (10 mL). The filtrate was dried over anhydrous $Na_2SO_4$ and solvent removed under aspirator pressure. The colorless product **5** (13.0 g, 54% yield) was distilled under reduced pressure (bp 65–66 °C (0.05 Torr)): $^1$H NMR (300 MHz, $CDCl_3$) δ 1.24 (s, 6 H), 1.84 (t, $J$ = 6.9 Hz, 2 H), 2.21 (s, 1 H), 4.03 (s, 2 H), 4.34 (t, $J$ = 6.9 Hz, 2 H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 167.23, 69.73, 63.17, 41.18, 36.95, 29.52; IR (neat film, cm$^{-1}$) 3508, 3424, 2973, 2937, 1739, 1470, 1414, 1377, 1317, 1188, 1000, 938; refractive index $n^{21}_D$ = 1.456; mass spectrum (FAB, *m*-nitrobenzyl alcohol matrix) calcd for $C_7H_{14}O_3Cl$ (M + H) 181.0631, found 181.0636. Compound **4** was prepared in an analogous fashion.

**[2-[2-[[3-(Chloracetoxy)-1,1-dimethyl-1-propoxy]dimethylsiloxy]ethoxy]ethoxy]ethyl]methyl Ether (CSTE) (8).** Neat dichlorodimethylsilane (0.72 g, 5.5 mmol) was added dropwise under a nitrogen blanket to a stirred solution of **5** (1.00 g, 5.5 mmol) and pyridine (0.52 g, 6.6 mmol) in 3 mL of ether at room temperature. After allowing displacement of the first chloride to run for 5 h a solution of triethyleneglycolmonomethyl ether (1.00 g, 6.1 mmol) and pyridine (0.60 g, 7.6 mmol) was added dropwise simultaneously from separate syringes and the reaction allowed to stir for another 5 h. The reaction mixture was then diluted with 50 mL of ether and filtered and the filtrate washed with 5 mL of water followed by 5 mL of brine. After being dried over $Na_2SO_4$ and removal of solvent under reduced pressure crude **8** was purified by flash chromatography on silica gel using 95:5 hexane/2-propanol: yield 1.52 g (69%); $^1$H NMR (300 MHz, $CDCl_3$) δ 0.13 (s, 6 H), 1.86 (t, $J$ = 7.2 Hz, 2 H), 3.38 (s, 3 H), 3.50–3.70 (m, 10 H), 3.81 (t, $J$ = 5 Hz, 2 H), 4.05 (s, 2 H), 4.34 (t, $J$ = 7.2 Hz, 2 H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 167.33, 72.95, 72.36, 71.92, 70.61, 70.52, 63.39, 61.59, 59.01, 42.49, 40.92, 30.06, −0.47; IR (neat film, cm$^{-1}$) 2971, 2927, 2902, 2874, 1758, 1736, 1318, 1291, 1256, 1174, 1144, 1104, 1021, 965, 949, 842, 792; mass spectrum (FAB, *m*-nitrobenzyl alcohol matrix) calcd for $C_{16}H_{34}O_7ClSi$ (M + H) 401.1762, found 401.1785. Compound **7** was prepared from **4** in an analogous fashion.

**CSTE-Papain (10).** A solution of purified papain was concentrated by centrifugation using a 10 kDa cutoff Centricon filter tube. A solution of **8** (0.5 mL, 4 mg/mL) in deionized water was added to 1 mL of concentrated papain (10.7 mg/mL) at 4 °C. After incubation for 2 h in a cold room, an aqueous solution of L-cysteine (0.05 mL, 20 mg/mL) was added to the reaction mixture, followed by a second addition of **8** (0.5 mL, 5 mg/mL). After incubation for 1.5 h more the unreacted CSTE and L-cysteine were removed by gel filtration on Sephadex G-25 followed by three consecutive dilution/concentration sequences. Enzymic activity of the modified protein **10** was ≤0.1% of the initial specific activity.

***S*-[2-[2-(2-Hydroxyethoxy)ethoxy]ethyl]cysteamine.** Neat 2-[2-(2-chloroethoxy)ethoxy]ethanol (10.6 g, 62 mmol) was added at once to a solution of 2-aminoethanethiol hydrochloride (6.8 g, 60 mmol) and suspended sodium bicarbonate (10.4 g, 124 mmol) in 160 mL of 1:1 (v/v) aqueous 1,4-dioxane. The reaction mixture was refluxed for 48 h with magnetic stirring, after which solvent was removed under aspirator pressure on a rotary evaporator. The residue was dissolved in $CHCl_3$ (100 mL) and filtered to remove inorganic salts. The filtrate was concentrated to a light green liquid, which was distilled under reduced pressure. The colorless product $H(OCH_2CH_2)_3SCH_2CH_2NH_2$ (7.1 g, 51% yield) was collected at 144–146 °C under 0.10 mmHg: $n^{21}_D$ = 1.505; $^1$H NMR (300 MHz, $CDCl_3$) δ 2.42 (bs, 3 H), 2.68 (m, 4 H), 2.87 (t, 2 H), 3.63 (m, 10 H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 72.97, 71.18, 70.32, 70.33, 61.40, 40.95, 37.08, 31.67; IR ($CCl_4$, cm$^{-1}$) 3607, 3375, 3308; mass spectrum (FAB, *m*-nitrobenzyl alcohol matrix) calcd for $C_8H_{20}NO_3S$ (M + H) 210.1164, found 210.1164.

***S*-[2-[2-(2-Hydroxyethoxy)ethoxy]ethyl]cysteamine Biotinamide.** A 3-neck flask was charged with 1,1′-carbonyldiimidazole (3.30 g, 20.46 mmol) and biotin (4.91 g, 20.1 mmol) in 50 mL of DMF under argon at room temperature. After 3 h, neat $H(OCH_2CH_2)_3SCH_2CH_2NH_2$ (4.48 g, 21.4 mol) was added dropwise with magnetic stirring, and stirring was continued for 24 hours, after which the solvent was removed under reduced pressure with a vacuum pump. The residue was dissolved in 100 mL of hot methanol, crystallized by adding 400 mL of ether (cooled with an ice bath), and recrystallized from 2-methyl-2-propanol/pentane (7.4 g, 80% yield): $^1$H NMR (300 MHz, $CDCl_3$) δ 1.47 (m, 2 H), 1.71 (m, 4 H), 2.24 (t, $J$ = 7.5 Hz, 2 H), 2.75 (m, 5 H), 2.93 (dd, $J$ = 4.8 and 13 Hz, 1 H), 3.15 (dt, $J$ = 4.5 and 7.2 Hz, 1 H), 3.45 (q, $J$ = 9 Hz, 2 H), 3.60–3.71 (m, 9 H), 3.75 (t, $J$ = 4 Hz, 2 H), 4.33 (m, 1 H), 4.52 (m, 1 H), 5.20 (s, 1 H), 6.16 (s, 1 H), 6.71 (t, $J$ = 5.1 Hz, 1 H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 25.54, 28.04, 28.07, 31.55, 32.43, 34.66, 35.82, 38.69, 40.56, 55.47, 60.13, 61.58, 61.77, 70.28, 70.31, 70.99, 72.52, 163.64, 173.29; IR ($CDCl_3$, cm$^{-1}$) 3468, 3446, 2928, 1708, 1664, 1516, 1439, 1428, 1332, 1258, 1118, 1062; melting point 124–125 °C; mass spectrum (FAB, *m*-nitrobenzyl alcohol matrix) calcd for $C_{18}H_{34}O_5N_3S_2$ (M + H) 436.1940, found 436.1964.

***S*-[2-[2-[2-[[3-(Chloroacetoxy)-1,1-dimethyl-1-propoxy]-dimethylsiloxy]ethoxy]ethoxy]ethyl]cysteamine Biotinamide (CSSB) (9).** Dichlorodimethylsilane (0.34 g, 2.6 mmol) was added to a solution of **5** (0.43 g, 2.4 mmol) and imidazole (0.18 g, 2.6 mmol) in DMF (3.0 mL) at room temperature under nitrogen and stirred for 12 h. A solution of $H(OCH_2CH_2)_3SCH_2CH_2NH$-biotin (0.84 g, 2.0 mmol) and imidazole (0.20 g, 3.0 mmol) in DMF (6 mL) was then added dropwise to the reaction mixture. After 6 h solvent was removed under reduced pressure with a vacuum pump and residues extracted with THF. After concentrating the THF solution, flash chromatography with 2-propanol/hexane (3:1) on silica gel was run to obtain product **9** (0.14 g, 10%). It proved to be impossible to remove all impurities by flash chromatography, and **9** was used for protein modification without additional purification: $^1$H NMR (300 MHz, $CDCl_3$) δ 0.13 (s, 6 H), 1.32 (s, 6 H), 1.46 (m, 2 H), 1.70 (m, 4 H), 1.86 (t, $J$ = 7.5 Hz, 2 H), 2.23 (t, $J$ = 7.5 Hz, 2 H), 2.73 (m, 5 H), 2.92 (dd, $J$ = 4.8 and 13 Hz, 1 H), 3.16 (dt, $J$ = 4.5 and 7.2 Hz, 1 H), 3.44 (q, $J$ = 6 Hz, 2 H), 3.59 (t, $J$ = 5 Hz, 2 H), 3.64 (m, 6 H), 3.81 (t, $J$ = 5.4 Hz, 2 H), 4.06 (s, 2 H), 4.35 (m, 3 H), 4.52 (m, 1 H), 5.06 (s, 1 H), 5.86 (s, 1 H), 6.42 (t, $J$ = 5.4 Hz, 1 H); mass spectrum (FAB, *m*-nitrobenzyl alcohol matrix) calcd for $C_{27}H_{51}O_8N_3S_2SiCl$ (M + H) 672.2575, found 672.2557.

**CSSB-Papain (11).** A solution of freshly purified papain was concentrated by centrifugation and degassed under vacuum by a freeze–pump–thaw cycle. A 5-fold excess of **9** in an equivalent volume of DMSO was slowly added at 4 °C. After incubation for 1 h in a cold room, the reaction mixture was diluted with an equal volume of 50 mM Tris buffer containing 10 mM cysteine at pH 7.5. A small amount of precipitate formed, which was removed by centrifugation, and unreacted CSSB was then removed by diluting the filtrate with buffer and concentrating using centrifugation in Centricon tubes. The dilution/concentration sequence was repeated to ensure removal of unreacted **9**. Enzymic activity of **11** was ≤2% of initial specific activity.

**Kinetic Studies.** Aqueous halide solutions (KF, KCl, and $Me_4NF$) were prepared in $D_2O$ buffered with 5 mM Tris at pD 7.4. Samples of $Ph_2Si[(OCH_2CH_2)_3OCH_3]_2$ were completely dissolved in buffered $D_2O$ by sonication before mixing with an equal volume of halide solution. The temperature was maintained at 22 ± 0.5 °C, and hydrolysis was monitored by proton NMR at 300 or 500 MHz. The digital integrals of the methoxy resonances of the starting material and the product as a function of time were used to calculate hydrolysis rates. Compound **3** gave no evidence of hydrolysis over 24 h under these conditions nor in the absence of fluoride at pD 5.5 or pD 10.1.

Hydrolysis kinetics of other model compounds in $D_2O$ solution were monitored by proton NMR in the same fashion. Chemical shifts are referenced to DSS. Multiple regression analyses were performed on a personal computer using the MINSQ program.[8]

Hydrolysis kinetics for modified papain 7 in 9:1 $H_2O/D_2O$ was monitored using a 1-3-3-1 NMR pulse sequence[9] to suppress the water signal.

Elution kinetics for two-step affinity chromatography were monitored as follows. A solution of 11 ($2.4 \times 10^{-5}$ M) in phosphate-buffered saline (0.02 M sodium phosphate, 0.15 M NaCl, pH 7.45) was added dropwise to a solution of 3 mL of streptavidin (1 mg/mL) at 4 °C in a polyethylene centrifuge tube and mixed by vortexing after every two drops. After addition was complete the mixture was incubated for 30 min and the absorbance at 280 nm determined. A portion of this solution was loaded onto a biotin-agarose affinity column and continuously washed with buffered 0.5 M KCl until the $OD_{280}$ of the eluent fell to zero. Approximately 30–40% of the modified protein was retained. Then the column was incubated with 0.05 M Tris (pH 7.5) containing potassium halide (either 0.5 M KCl, 0.1 M KF, 0.2 M KF, or 0.5 M KF) and the $OD_{280}$ of subsequent washings after each incubation interval was followed as a function of time.

## Results

In order to study hydrolysis in a homogeneous aqueous medium, it was necessary to synthesize water-soluble dialkoxysilanes. The simplest example was prepared as eq 1 depicts,[7] by using the monomethyl ether of ethylene

$$PhSiRCl_2 + \underset{\mathbf{1}}{2R'OH} \xrightarrow[\text{benzene}]{\text{pyridine}} \underset{\begin{array}{l}\mathbf{2},\ R = Ph\\ \mathbf{3},\ R = t\text{-Bu}\end{array}}{PhSiR(OR')_2} \quad (1)$$

$$R' = -(CH_2CH_2O)_3CH_3$$

glycol (1) to provide both alkoxy moieties. The solubility of $Ph_2Si[O(CH_2CH_2O)_3CH_3]_2$ (2) is on the order of 1 mM in water at pH 7, a concentration high enough to permit monitoring of hydrolysis kinetics by proton NMR. As in previously reported studies[10] (both single-phase and two-phase), cleavage of silicon ethers is catalyzed both by acid and by base, with a rate minimum near neutrality. We have examined kinetics in $D_2O$ buffered with 5 mM $N(CH_2OH)_3$ (Tris) at pD 7.4 (which corresponds to a reading of 7.0 on a standard glass electrode pH meter) at 295 K. Under these conditions, with no added fluoride, the half-life of 2 is 22 h. Concentrations of reactant 2 and its hydrolysis products, 1 and dihydroxydiphenylsilane, can be measured by NMR integration. No other resonances apart from these and those of the buffer are observed in the 500-MHz proton NMR during the course of reaction. The methyl resonances of 1 (singlet at $\delta$ 3.391) and 2 (singlet at $\delta$ 3.349) are easily resolved and integrated. Under acidic or basic conditions hydrolysis of 2 is faster than at neutrality, with a half life of 10 h at pD 5.5 and a half life of 8 min at pD 10.1.

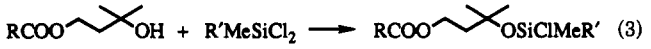
When potassium fluoride is added at pD 7.4 the hydrolysis rate increases with fluoride concentration. Hydrolysis of 2 obeys pseudo-first-order kinetics under all conditions in the range of KF concentrations from 0 to 100 mM. The observed rate constant, $k_{obs}$, is well fit as a linear function of potassium fluoride concentration, [KF]: $k_{obs} = 9 \times 10^{-6}\ s^{-1} + k[KF]$. The first term represents the first-order hydrolysis rate constant, $k_{D_2O}$, at pD 7.4 in the absence of fluoride. The second term corresponds to the fluoride-catalyzed component, for which $k = 4.3 \times 10^{-3}\ M^{-1}\ s^{-1}$. If the activities of $F^-$ for KF solutions in water, $a_{F^-}$, are used in place of [KF], the second term becomes $5.6 \times 10^{-3}\ a_{F^-}$ (standard deviation = $0.1 \times 10^{-3}$). The cation appears to play a negligible role in the catalysis. In buffered 50 mM KCl the observed hydrolysis rate constant is within experimental error of $k_{D_2O}$, while the catalyzed rate constant in 50 mM tetramethylammonium fluoride is no less than its value in 50 mM KF.

Corriu and co-workers have described a mechanism for fluoride catalysis of displacement at silicon in which the first step involves a pentacoordinate intermediate.[11] Water would then attack this intermediate in a rate-determining step (RDS) to yield a hydroxyalkoxysilane, for which further hydrolysis is presumed to be fast. Our results are not inconsistent with such a mechanism, although pseudo-first-order kinetics fit data at least as well. If we assume hydrolysis of the pentacoordinate intermediate to be rate limiting, the corresponding rate law will be

$$k_{obs} = k_{D_2O} + [ka_{F^-}/(a_{F^-} + K_d)] \quad (2)$$

A double reciprocal plot gives $K_d = 0.6$ M and $k = 3.4 \times 10^{-3}\ s^{-1}$ ($r^2 = 0.98$).

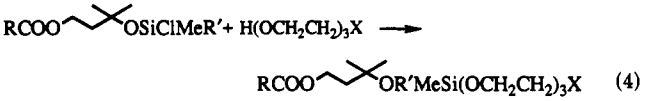
Unsymmetrical dialkoxysilanes were prepared as shown by eqs 3 and 4. Tertiary alcohols react with dichlorosilanes to displace only one chlorine, as eq 3 depicts, forming an



(3)

**4** R=$CH_3$

**5** R=$CH_2Cl$



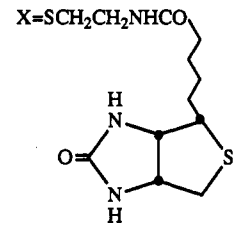
(4)

**6** R=$CH_3$, R'=Ph, X=$OCH_3$

**7** R=R'=$CH_3$, X=$OCH_3$

**8** R=$CH_2Cl$, R'=$CH_3$, X=$OCH_3$

**9** R=$CH_2Cl$, R'=$CH_3$,




alkoxychlorosilane that is very slow to react with a second molecule of tertiary alcohol. Subsequent addition of a primary alcohol results in substitution of the remaining chloride, as portrayed in eq 4.

Compounds 6–9 were prepared in this fashion. The presence of hydrophilic chains containing $-OCH_2CH_2-$ links renders them somewhat soluble in water, and the hydrolysis kinetics of compounds 6 and 7 were examined in aqueous solution. With a *tert*-alkoxy group attached to silicon the rate of hydrolysis is slowed down considerably by comparison to silicon to which two primary alkoxy groups are attached. The methylphenylsilyl compound 6 hydrolyzes at a rate slower than $[CH_3(OCH_2CH_2)_3O]_2SiPh_2$. Under conditions of fluoride ca-

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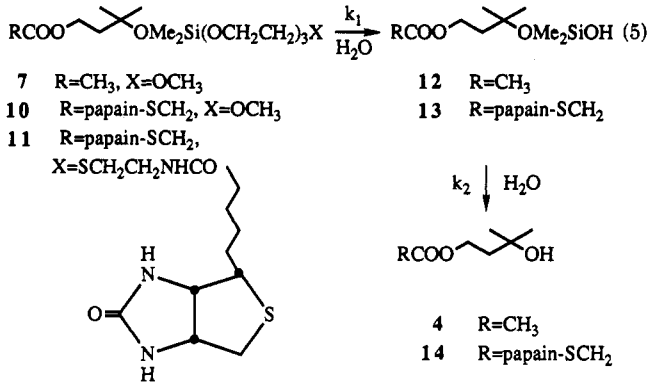
(11) (a) Corriu, R. J. P.; Guerin, C. *Adv. Organomet. Chem.* **1982**, *20*, 265–312. (b) Corriu, R. J. P.; Dutheil, J.-P.; Lanneau, G. F. *J. Am. Chem. Soc.* **1984**, *106*, 1060–1065.

talysis the first step, hydrolysis of the primary alkoxy–silicon bond, occurs much faster than the hydrolysis of the *tert*-alkoxy–silicon bond. This stands in contrast to the case of symmetrical dialkoxysilanes, where the second step is faster than the first.

By following hydrolysis kinetics of unsymmetrical dialkoxysilanes by NMR we can resolve two distinct hydrolysis rates, $k_1$ and $k_2$, corresponding to cleavage of the primary and *tert*-alkoxy–silicon bonds, respectively, as eq 5 represents. The hydrolysis steps can be monitored by




the disappearance of the $(CH_3)_2Si$ singlet of the starting material and the appearance of a new $(CH_3)_2Si$ singlet, followed by a third singlet. This third singlet increases with time at the expense of the other two, and the rates can be fitted by pseudo-first-order kinetics. Because a single cleavage is sufficient for the purposes of the two-step affinity chromatography protocol we have focused on the dependence of $k_1$ upon catalyst concentration.

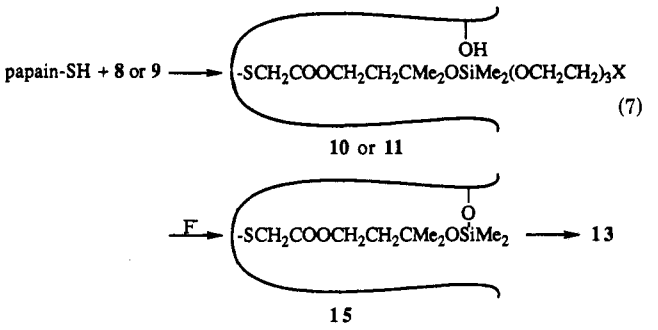
As models for hydrolysis of unsymmetrical dialkoxysilanes, kinetics of **6** and **7** were examined in $D_2O$ solution. Two steps of hydrolysis can be seen, as eq 5 represents. At neutral pH, the half-life of **6** is 260 h with regard to hydrolysis of the primary alkoxy–silicon bond. The resulting intermediate has a half-life of 165 hours with respect to hydrolysis of the tertiary alkoxy–silicon bond. In the presence of 0.1 M KF both half lives are shortened, to 20 h for the first hydrolysis step and 65 h for the second. Thus, both hydrolysis steps are catalyzed by fluoride ion, but the first step is more sensitive to this catalysis than the second.

The hydrolysis kinetics of **7** were studied in greater detail. The half life of **7** at neutral pH buffered with 5 mM Tris in the absence of fluoride is on the order of 175 h. In the absence of fluoride $k_1^{obs}$ can be compassed by two terms, specific acid catalysis ($k_H = 0.11\ M^{-1}\ s^{-1}$) and general acid catalysis by buffer ($k_{Tris\cdot H} = 5.0 \times 10^{-4}\ M^{-1}\ s^{-1}$). In the presence of fluoride there are an additional second-order term ($k_F = 1.2 \times 10^{-4}\ M^{-1}\ s^{-1}$) and a third-order term ($k_{HF} = 6.5\ M^{-2}\ s^{-1}$), as shown in eq 6. Introducing addi-

$$k_1^{obs} = k_H[a_{H^+}] + k_{Tris\cdot H}[Tris\cdot H^+] + k_F[a_{F^-}] + k_{HF}[a_{F^-}][a_{H^+}] \quad (6)$$

tional parameters (such as replacing the third term of eq 6 with $ka_{F^-}/[a_{F^-} + K_d]$) does not improve the quality of the fit to the experimental data, since values for the dissociation constant always turn out to be $K_d > 3$ M.

We have prepared the chloroacetyl ester **8** and used it to modify the cysteine-protease papain. Papain is a monomeric protein with a sulfhydryl group (cysteine 25) at its active site. Chloroacetates are well-known to carboxymethylate this group through nucleophilic displacement of chloride by the thiol, as represented in the first step of eq 7. Once formed, the covalent adduct can be monitored




by the proton NMR of the $SiMe_2$ group, which appears as a singlet at $\delta$ 0.24. The *gem*-dimethyls attached to carbon, however, cannot be discerned. The covalent adduct **10** is labile with respect to hydrolysis ($t_{1/2}$ = 5 h in 90% $H_2O$:10% $D_2O$ at pH 7.4 at 25 °C). Addition of fluoride ion accelerates the hydrolysis of the silicon–oxygen linkage. In the presence of 0.1 M KF the disappearance of **10** exhibits pseudo-first-order kinetics $k = 1.6 \times 10^{-4}\ s^{-1}$. The $SiMe_2$ resonance of **10** disappears and a new singlet appears at $\delta$ 0.19 followed by a third singlet at $\delta$ 0.17. The resonance at $\delta$ 0.19 disappears as the resonance at $\delta$ 0.17 grows until it is the only $SiMe_2$ resonance observed. Concomitant with the disappearance of the first resonance (and the appearance of the other two) a singlet at $\delta$ 1.245 appears in the proton NMR, which we assign to the $CMe_2$ *gem*-dimethyl group. The second step is faster than the first, which, combined with the poor signal-to-noise for the small singlets superimposed on a protein NMR spectrum, prevented our measuring the value of $k_2/k_1$ accurately. The uncertainty in determining the rate constant for the appearance of the $CMe_2$ singlet likewise does not permit us to assess whether it corresponds to both the intermediate and the final product or to the final product alone.

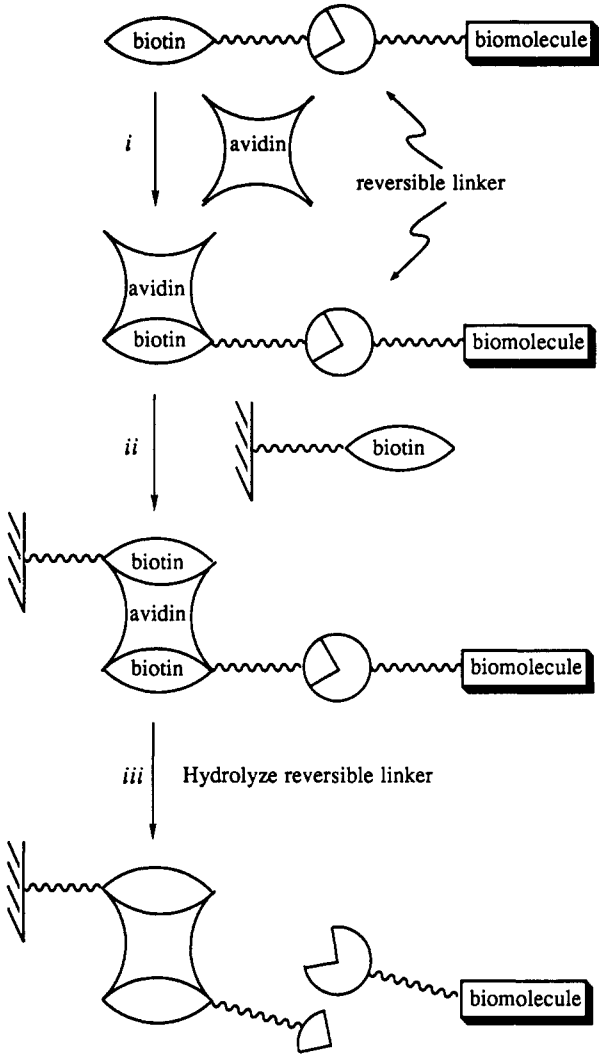
We can interpret this result in either of two ways. The hydrolysis could proceed via the two steps shown in eq 5. If this is the case, then the intermediate $SiMe_2$ resonance observed during the kinetic run corresponds to the species **13**, which then hydrolyzes to **14** plus $Me_2Si(OH)_2$. An alternative possibility supposes that a reactive residue (such as serine 176, which is within a few Å of the active site cysteine[12]) participates in the first fluoride-catalyzed step. This possibility is illustrated by the second step in eq 7, and it could account for the lability of **10** as compared to **7** (since hydrolysis of **7** is not affected by the presence of high concentrations of added papain). The second hydrolysis step would then correspond to hydrolysis of **15** to yield **13**, which might be immune to further hydrolysis because the O–Si bond is sequestered within the active site.

The principal evidence for the mechanism in eq 7 is that the final product of hydrolysis (after small molecules were removed by two dilution/concentration steps in a Centricon filter tube) is a protein that exhibits $CMe_2$ and $SiMe_2$ resonances in the proton NMR with approximately equal areas. We infer that the final product of hydrolysis must therefore be **13** and that the chain is mobile enough for these singlets both to have line widths of 2 Hz. The results of two-step chromatography suggest that a mechanism such as eq 7 also operates when the tether spans two macromolecules.

Covalent modification of papain with the biotinylated chloroacetate ester **9** affords the modified protein **11**, which can be attached to biotin-agarose, as depicted in either Scheme III or Scheme IV, or to avidin-agarose. We have

(12) Drenth, J.; Jansonius, J. N.; Koekoek, R.; Wolthers, B. G. *Adv. Protein Chem.* **1971**, *25*, 79–116.

**Scheme IV**




studied the elution of the modified papain attached under all three conditions, using both egg white avidin and streptavidin, but we shall focus on the results using Scheme IV with streptavidin. Uncatalyzed hydrolysis of the tether leads to gradual elution of the bound protein even in the absence of catalyst. This nonselective elution depends on temperature, as summarized by the lower dashed and solid curves in Figure 1. Uncatalyzed elution in 0.5 M KCl obeys first-order kinetics: $k = 1.7 \times 10^{-5}$ s$^{-1}$ at 24 °C and $3.8 \times 10^{-6}$ s$^{-1}$ at 4 °C.

Addition of fluoride to **11** bound to agarose-biotin-streptavidin accelerates the elution of modified papain, as the curves in Figure 1 summarize. This elution, too, is temperature dependent. The elution profiles at 24 °C (dashed curves for 0.5 M KF, 0.1 M KF, and 0.5 M KCl; individual data points not shown) clearly follow pseudo-first-order kinetics. The elution profiles at 4 °C (solid curves, with data points represented by closed symbols) are, however, only approximately fitted by pseudo-first-order kinetics. Addition of detergent to the eluent has no effect on the rate of elution (representative data points shown as open symbols). We speculate that fluoride may not diffuse rapidly into the active site and that its effective concentration in the vicinity of silicon is not constant on the time scale of elution. In any event, the elution product exhibits two singlets ($\delta$ 0.17 and 1.24) that are not present in native papain. Figure 2 displays the relevant region of the proton NMR spectrum of the modified papain purified by two-step affinity chromatography, to which we assign

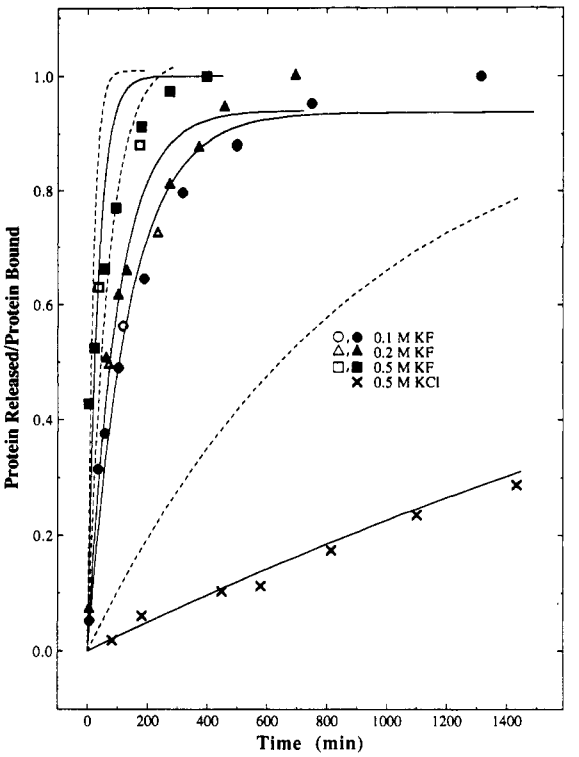



**Figure 1.** Release of modified papain **13** connected via streptavidin to a biotin-agarose column as a function of time for various buffered (0.05 M Tris, pH 7.5) aqueous potassium halide eluents at 24 °C (dashed lines) and 4 °C (solid lines). Individual data points are shown for only the 4 °C curves, with open symbols corresponding to representative data points for detergent-containing (0.1% Triton X-100) eluent and closed symbols for detergent-free eluents.

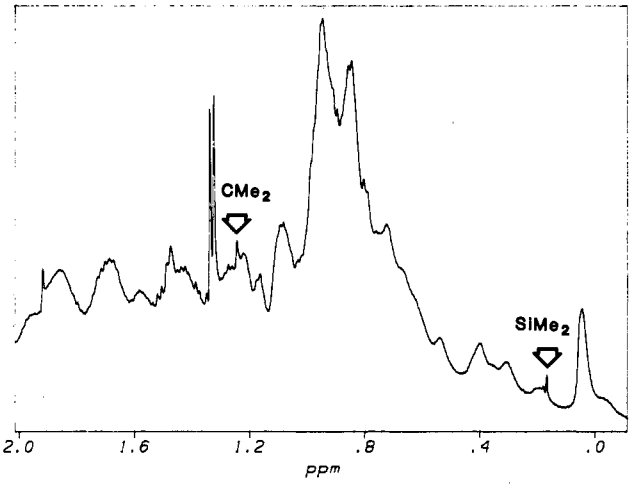



**Figure 2.** A portion of the 500-MHz proton NMR spectrum (in $D_2O$ at pD 8.2) of eluted modified papain from two-step affinity chromatography of **13**. Indicated peaks correspond to remnants of the tether not present in native papain.

the structure **13**. Isoelectric focusing gel electrophoresis shows that this protein runs as a single band with the same pI as native papain.

## Discussion

Two-step affinity chromatography has been reduced to practice, as portrayed in Schemes III and IV. This involves covalent attachment of biotin to the protein of interest via a hydrophilic tether that contains a fluoridolyzable linker. The first step is immobilization of the modified protein with streptavidin onto a solid support to which another molecule of biotin is attached. The second step is selective

release by fluoride-catalyzed hydrolysis of the fluoridolyzable linker (a $-CH_2OSiMe_2O-$ moiety within the tether).

The kinetics of hydrolysis of model compounds with hydrophilic chains, both a symmetrical example containing a $-CH_2OSiPh_2OCH_2-$ unit and an unsymmetrical example containing a $-CH_2OSiMe_2OCMe_2-$ unit, have been examined. The hydrolysis proceeds via cleavage of the primary alkoxy–silicon linkage to sever the tether. Hydrolysis in homogeneous media obeys a pseudo-first-order rate law. The phenomenological rate constant for hydrolysis of the $CH_2O$–Si bond of compound **7**, $k_1^{obs}$, at pH values near neutrality is fitted (albeit not perfectly) by an expression with four terms: specific acid catalysis, general acid (buffer) catalysis, catalysis by fluoride, and combined catalysis by fluoride and acid together.

Hydrolysis of simple organic compounds such as **7** proceeds in two discrete, observable stages, cleavage of the $CH_2O$–Si bond followed by cleavage of the Si–$OCMe_2$ bond. Hydrolysis of the tether attached to papain (**10**), however, yields a protein that still has an $SiMe_2$ group attached to it. While the hydrolysis obeys a pseudo-first-order rate law in homogeneous solution with two detectable stages, we surmise that the mechanism may operate as depicted in eq 7.

Liberation of tethered papain from a biotin-avidin affinity column is efficiently catalyzed by fluoride. Elution is up to 1 order of magnitude faster in the presence of fluoride than in its absence, and there is no evidence for saturation kinetics, even at fluoride concentrations as high as 0.5 M. The kinetics of fluoride-catalyzed release at 4 °C, however, are only approximately fitted by pseudo-first-order kinetics, as can be seen by comparing the solid symbols with the solid lines. Here we speculate that diffusion of $F^-$ is not rapid on the time scale of hydrolysis of the fluoridolyzable linker and that $a_{F^-}$ at the site of cleavage changes during the course of elution. The modified papain recovered from two-step affinity chromatography shows singlets in the proton NMR corresponding to a $SiMe_2$ and a $CMe_2$ group, and we infer that a silicon-containing remnant of the tether must remain attached to the protein (corresponding to **13**). Since these resonances are not broad (2-Hz line widths) we conclude that this remnant must be mobile within the active site to which it is attached.

These experiments illustrate the potential utility of two-step affinity chromatography, which incorporates the following novel features: use of a hydrophilic tether and incorporation of a fluoridolyzable linker. We have previously noted the advantages of connecting biotin via a hydrophilic molecular tether,[13] wherein the chain can be expected to be fully extended in aqueous solution. Use of a fluoridolyzable linker allows the elution step to be achieved with a reagent ($F^-$) that does not covalently alter amino acid residues (as is the case when the cleavable unit is a *vic*-diol or disulfide linkage[5]). While we cannot yet claim to understand fully all the mechanistic features of fluoridolyzable linkers in heterogeneous media (such as in the present case, where the tether connects one macromolecule to another one that is, in turn, bound to a surface), the practical aspects of this approach have been demonstrated.

## Conclusion

Two-step affinity chromatography designates, in general, a procedure that involves (1) specific binding of a biomolecule to a solid support (e.g., via biotin/streptavidin/biotin coupling) followed by (2) selective elution using a chemically specific cleaving agent that severs the tether attached to the biomolecule. We view this as a procedure that can potentially be used in conjunction with two-step covalent modification of proteins[14] in order to isolate cell-surface receptors from tissue samples. Other possible applications include reversible attachment of pairs of macromolecules in homogeneous solution in order to investigate proximity effects. Efforts to exploit this technology are underway.

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**Registry No.** 1, 109-86-4; 2, 58255-81-5; 3, 136805-95-3; 4, 5205-01-6; 5, 136805-96-4; 6, 136805-97-5; 7, 136805-98-6; 8, 136805-99-7; 9, 136806-00-3; $H(OCH_2CH_2)_3OCH_3$, 112-35-6; $H(OCH_2CH_2)_3SCH_2CH_2NH_2$, 136806-03-6; diphenyldichlorosilane, 80-10-4; phenyl-*tert*-butyldichlorosilane, 17887-41-1; 3-methyl-1,3-butanediol, 2568-33-4; chloroacetyl chloride, 79-04-9; dichlorodimethylsilane, 75-78-5; 2-[2-(2-chloroethoxy)ethoxy]-ethanol, 5197-62-6; 2-aminoethanethiol hydrochloride, 156-57-0; biotin, 58-85-5; *S*-[2-[2-(2-hydroxyethoxy)ethoxy]ethyl]cysteamine biotinamide, 136806-04-7.

**Supplementary Material Available:** Proton and carbon NMR spectra of new compounds and tabulated kinetic data (20 pages). Ordering information is given on any current masthead page.

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