Partial Purification of the 5-Hydroxytryptophan-Reuptake System from Human Blood Platelets Using a Citalopram-Derived Affinity Resin†

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ABSTRACT: This paper describes a procedure for the synthesis and application of a citalopram-derived affinity resin in purifying the 5HT-reuptake system from human blood platelets. A two-step scheme has been developed for partial purification, based on wheat germ agglutinin–lectin (WGA) affinity and citalopram affinity chromatographies. Upon solubilization of the carrier with 1% digitonin, a 50-70-fold increase in specific [3H]imipramine binding activity with a 70% recovery could be accomplished through WGA–lectin chromatography. The WGA pool was then subjected to affinity chromatography on citalopram–agarose. At least 90% of the binding capacity was adsorbed to the column. Specific elution using 10 μM citalopram resulted in a 22% recovery of binding activity. A 10 000-fold overall purification was obtained by using this two-step procedure. Analysis of the fractions on SDS–PAGE after [3H]elution revealed specific elution of 78- and 55-kDa proteins concomitant with the appearance of [3H]imipramine binding activity. The pharmacological profile of the partially purified reuptake system correlated well with that derived from the crude membrane-bound reuptake system, suggesting a copurification of the 5HT binding activity and [3H]imipramine binding activity.

The 5HT-reuptake system has been well documented, during the past decades, by both radioligand binding and 5HT-transport studies (Davis, 1984). Substantial evidence has been furnished supporting the concept that the 5HT-reuptake system involves closely interacting sites, a carrier site and a regulatory imipramine binding site (IBS).1 Binding of reuptake inhibitors to the regulatory site strongly inhibits 5HT reuptake. The structural relationship between the transport and regulatory sites is unclear (Humphreys et al., 1988; Biessen et al., 1988; Reith et al., 1984; Mellerup et al., 1983; Sette et al., 1983). Despite the fact that 5HT reuptake appears to be more or less clarified on a macroscopic level, the molecular basis of transport and its regulation are only poorly understood. The molecular masses of the functional imipramine and paroxetine binding sites were estimated by using radiation inactivation studies to be 86 and 67 kDa, respectively (Mellerup et al., 1984). By contrast, photoaffinity labeling of the IBS using [3H]-2-nitroimipramine and [3H]-2-azidoimipramine revealed incorporation of the photoaffinity label in a 30–35-kDa protein (Wennogle et al., 1985; Rotman & Prídluda, 1982). Whether the labeling was specific has not been convincingly demonstrated.

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‡A. S. Horn died on January 2, 1990, as a result of injuries sustained in an automobile accident 2 days earlier. Those of us who had the pleasure of knowing him and working with him will miss him dearly.

Abbreviations: AMC, (aminomethyl)citalopram; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; Con-A, concavalin A; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; EI, electron impact; EG, ethylene glycol; IBS, imipramine binding site; KAc, potassium acetate; KP, potassium phosphate; NaSCN, sodium thiocyanate; PAA, polyacrylamide; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; WGA, wheat germ agglutinin.
Solubilization of the 5HT-reuptake system from both thrombocytes and brain tissue with digitonin, CHAPS, or lyssolecithin to yield a preparation retaining most of the characteristics of the crude reuptake system has been reported by various research groups (Talvanheimo & Rudnick, 1980; Habert et al., 1986; Cesura et al., 1983; Rehavi et al., 1982). In view of its minute density and the high affinity of most selective reuptake inhibitors for the transport system, affinity chromatography appeared to be the method of choice in isolating the system. In this paper we present the synthesis of a citalopram-derived affinity resin and a two-step purification scheme using WGA–lectin and citalopram affinity chromatographies which allow a 10 000-fold purification. The pharmacological profiles of the purified protein and the crude membrane-bound or solubilized reuptake system are essentially the same, suggesting a copurification of the 5HT and imipramine binding activity.

**METHODS**

**Tissue Preparation.** The platelet membranes were prepared by a modification of the procedure of Mellerup et al. (1983) as previously reported (Biessen et al., 1988). After preparation of the platelet membranes, the tissue was suspended in solubilization buffer (250 mM NaSCN, 50 mM Tris-HCl, 5 mM KCl, 1 mM EDTA, 2 mM MgCl2, 0.3 mM DTT, 0.1 mM PMSF, pH 7.5) at a protein concentration of 15 mg/mL and the membranes were solubilized by incubation with an equal volume of 2% digitonin in solubilization buffer for 60 min at 4 °C under mild agitation. After centrifugation (60 min at 100 000 g), the supernatant was decanted and stored in liquid nitrogen at a protein concentration of about 5–7 mg/mL.

**[3H]Imipramine Binding Assay.** Radioligand-binding assays were performed according to the method previously reported (Biessen et al., 1988). Displacement binding studies were performed at a fixed concentration of 4 nM [3H]imipramine in the presence of unlabeled displacer, at 10 concentrations or 7 for the partially purified preparation) ranging from 0.1 nM to 100 nM. Following incubation with [3H]imipramine, the reuptake system was rapidly precipitated by adding 100 μL of 0.3% bovine γ-globulin immediately followed by 200 μL of 30% PEG 6000. After the precipitate was dried under vacuum to give 390 mg of product (78%): mp 186–190 °C; mass spectrometry (EI) m/z 328 (M+), 283, 242, 84; IR (KBr) 3415, 2950, 2860, 1606, 1507, 1465, 1230, 1168, 1040, 1023, and 839 cm⁻¹; 1H NMR (D₂O) δ 6.9–7.6 (7 H, m), 2.90 (6 H, s), 2.29 (2 H, t), 1.85 (2 H, q). A single spot on TLC, using EtOAc/EtOH (1:1), was observed.

**Preparation of AMC-Affi-Gel.** Coupling of the citalopram derivative was achieved as follows. AMC-2HCl (700 mg) was dissolved in 25% DMSO and 25% 1,4-dioxane in KPi buffer (50 mM, pH 7.0). The solution was added to 15 mL of preswollen NHS-activated Sepharose (Affi-10), and the mixture was rotated at room temperature for 4 h. Next, the gel slurry was incubated overnight at 4 °C under constant agitation, and the slurry was washed batchwise with a dioxane/H₂O mixture decreasing from 50% 1,4-dioxane to pure water. The unreacted, free NHS groups were quenched by incubation for 2 h with 1 M ethanolamine at room temperature. Finally, the gel slurry was washed thoroughly with 500 mL of SDS, 250 mL of water, 25%, 50%, 75%, 50%, and 25% ethanol and water, and finally 1 L 0.1 M KAc buffer (pH 4.0) to remove all free ligand. Even with this extensive washing procedure, some batches of AMC–Affi-gel still contained sufficiently high amounts of free AMC to prevent adsorption of binding activity to the AMC–Affi-Gel the first time that the gel was used after synthesis.

**Iodination with Chloramine T.** Prior to gel electrophoresis, the affinity-purified samples were iodinated with Na¹²⁵¹I by using Chloramine T. In short, a 50-μL sample was reacted with 5 μL of Na¹²⁵¹I (100 μCi) for 1 min at room temperature...
in the presence of 5 μL of chloramine T (1 mg/mL in H₂O). The iodination was terminated by addition of 5 μL of sodium metabisulfite (1 mg/mL in H₂O). Following treatment with 65 μL of denaturation buffer at room temperature for 30 min, 50-μL samples were subjected to gel electrophoresis on 10–12.5% polyacrylamide gel according to the method of Laemmli (1970). Low molecular weight markers (Pharmacia) at concentrations of 50 ng of protein/mL each, and two BSA standards, at concentrations ranging from 50 to 5 ng/mL, were analogously iodinated and subjected to SDS–PAGE to calibrate the gel and to quantify the intensity of the visualized proteins. After running, the gel was washed for 1 h with 50% MeOH and dried under vacuum at 65 °C, and 125I-labeled proteins were visualized by using autoradiography on Kodak-X-Ormat for 1–3 days at ~70 °C.

**Data Calculation and Statistical Analysis.** Saturation curve data were subjected to a nonlinear regression program (Graph-Pad) fitting saturation according to a single-site binding model to evaluate K_d and B_max values. Displacement binding data were analyzed according to a single-site displacement binding model using a computerized nonlinear fitting program (Graph-Pad) to calculate the K_d and the apparent Hill coefficient.

**Materials.** All chemicals were of reagent grade. The re-uptake inhibitors were generous gifts from their respective firms: imipramine and clomipramine from Ciba-Geigy Lab (Bazel, Switzerland); citalopram and clonipramine from Lundbeck (Copenhagen, Denmark); zimelidine from Astra (Sweden); ORG 6582 from Organon (Oss, Holland); and fenoxetin from Ferrosan (Denmark). Affi-Gel 10 was purchased from Bio-Rad (Richmond, CA) and digitonin from Fluka (Buchs, Switzerland). [3H]Imipramine was purchased from New England Nuclear (Boston, MA; 52.6 and 45.3 Ci/mmol). Outdated platelet-rich plasma was kindly supplied by the Bloodbank, Groningen, The Netherlands.

**RESULTS**

**Solubilization of the IBS.** The IBS can be solubilized with digitonin. The summed activities of supernatant and the pellet directly reveal that digitonin can be used without loss of total activity at concentrations up to 1% digitonin (Figure 1). Protein is extracted from the membranes, even at 0% digitonin, possibly owing to the chaotropic action of sodium thiocyanate. Protein extraction leveled off at 1% digitonin. The solubilization yield increases at digitonin concentrations higher than 0.6% (Figure 1).

**TABLE I: Inhibition Constants for Various Reuptake Inhibitors and 5HT**

<table>
<thead>
<tr>
<th>displacer</th>
<th>platelet membranes</th>
<th>solubilate</th>
<th>partially purified receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>5HT</td>
<td>5.60 ± 0.1</td>
<td>5.77 ± 0.08</td>
<td>5.09 ± 0.23</td>
</tr>
<tr>
<td>imipramine</td>
<td>7.90 ± 0.2</td>
<td>8.02 ± 0.08</td>
<td>8.70 ± 0.17</td>
</tr>
<tr>
<td>2-nitroimipramine</td>
<td>7.90 ± 0.26</td>
<td>8.09 ± 0.07</td>
<td>ND</td>
</tr>
<tr>
<td>citalopram</td>
<td>8.30 ± 0.3</td>
<td>7.84 ± 0.07</td>
<td>8.49 ± 0.06</td>
</tr>
<tr>
<td>zimelidine</td>
<td>6.25 ± 0.04</td>
<td>5.91 ± 0.04</td>
<td>6.12 ± 0.23</td>
</tr>
<tr>
<td>ORG 6582</td>
<td>6.3 ± 0.17</td>
<td>6.12 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>fenoxetin</td>
<td>7.8 ± 0.05</td>
<td>7.74 ± 0.77</td>
<td>7.77 ± 0.18</td>
</tr>
<tr>
<td>clomipramine</td>
<td>8.2 ± 0.35</td>
<td>8.18 ± 0.11</td>
<td>8.74 ± 0.15</td>
</tr>
<tr>
<td>AMC</td>
<td>6.33 ± 0.16</td>
<td>6.36 ± 0.07</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Competition experiments were performed, in triplicate, at a fixed ligand concentration of approximately 4 nM [3H]imipramine for 7 displacer concentrations, in the case of studies on a partially purified 5HT-reuptake system, and for 10 displacer concentrations, in the case of solubilize and crude membranes. pK_i values were calculated from inhibition curve data by using a computerized nonlinear fitting procedure (Graph-Pad). ND indicates not determined. The correlation coefficients were 0.982 and 0.972 for partially purified vs crude and partially purified vs solubilized tissue, respectively.

0.2% and is optimal at 1% digitonin. Under these optimal conditions, the solubilization yield is 62 ± 4% (414 ± 40 fmol/mg of protein). Solubilization does not significantly affect the dissociation constant for imipramine; it was 2.47 ± 0.34 and 1.84 ± 0.4 nM for solubilize and crude platelet membranes, respectively. [3H]Imipramine binding kinetics on the solubilize were in close agreement with the data on the crude membrane-bound system. The pharmacological binding profile of the solubilized IBS essentially matched that of the crude membrane-bound receptor (Table I; r = 0.987).

**Testing of the Citalopram-Derived Affinity Resin.** AMC–Affi-Gel (Figure 2A) was selected from a series of 15 affinity resins based on structural analogues of imipramine, citalopram, and serotonin. About 5.3 μmol of AMC/g of wet resin was incorporated as determined by the extinction of hydrolyzed AMC–Affi-Gel at 260 nm. Preliminary studies showed that AMC–Affi-Gel adsorbed most of the binding activity (≥70%) and that the adsorbed binding activity could be recovered by specific elution with 10 μM citalopram. Preincubation of the solubilize with 1 μM citalopram prior to chromatography largely prevented retention of binding activity; thus establishing the specific nature of adsorption of the binding activity to the column. In the case of preblocking, 50 ± 15% of the binding
activity eluted from the column in the flow-through and the first wash step, relative to only 7 ± 5% in a control preparation which was not preblocked with citalopram (n = 2). Subsequent specific elution with 10 μM citalopram resulted in desorption of approximately 49 ± 15% (n = 2) of the applied binding activity for the control experiment, but only 4% in the case of preblocking with citalopram.

Purification. In a typical purification procedure, 10 mL of solubilize, containing approximately 25 pmol of binding capacity, was subjected to a prepurification step on WGA-Sepharose to reduce the digitonin level and to eliminate some proteins or phospholipids that might interfere with the affinity chromatography step. As demonstrated in Figure 3, most of the protein eluted from the column in the flow-through, whereas more than 88 ± 4% (n = 8) of the binding capacity was retained. Elution of the column with an N-acetylglucosamine gradient resulted in 70 ± 6% (n = 8) recovery of binding activity. SDS-PAGE analysis of the eluted fractions confirmed the large purification factor realized by this step; it was estimated to be 50-70-fold, on the basis of both the E_{280} profile and scanning of the PAA gels. The WGA pool was directly incubated with AMC-Affi-Gel. A column was packed and washed by using the preparative procedure given under Methods. Directly following the washing steps, binding activity was eluted by using 10 μM citalopram. Figure 4 shows an elution profile of a typical experiment. Almost all the binding activity of the prepurified solubilize is retained by the affinity column (91 ± 2%; n = 8). Binding activity can be recovered in part (22 ± 4%) upon elution with 10 μM citalopram. Analysis of the citalopram-eluted fractions on SDS-PAGE after labeling of the proteins with Na^{251}I revealed the appearance of 78- and 55-kDa proteins concomitantly with recovery of binding activity (Figure 5). The 78-kDa region appears to contain two bands; the lower of the two correlates with the presence of imipramine binding activity. The purity of the preparation was estimated to be 5-10% on the basis of SDS-PAGE analysis.

The pharmacological profile of the partially purified transporter was examined by using displacement binding studies of six typical reuptake inhibitors. K_{i} values of these compounds were comparable with values obtained by using crude platelet membranes, r = 0.982, or solubilize, r = 0.972 (Table I), suggesting a purification of the intact 5HT trans-
Hydroxytryptophan-Reuptake System from Human Platelets

Rehavi et al., 1982). Digitonin at a concentration of 1% results in the solubilization of at least 60% of the 5HT-reuptake system. Sodium thiocyanate stabilizes the complex during the solubilization relative to NaCl and slightly improves the solubilization yield, possibly by a chaotropic effect. The binding characteristics of the solubilized reuptake system closely resemble those from the original membrane-bound system.

A series of 15 affinity resins, varying from each other in ligand type (imipramine, citalopram, and serotonin derivatives), ligand orientation, spacer length (ranging from 5 to 20 Å), and covalent coupling (amine, amide, ether, ester bond), were synthesized. The serotonin-derived affinity resins displayed only moderate affinity for the reuptake system, leading to desorption of adsorbed proteins during the wash steps. A modest retention of binding activity was observed on chromatography over a 2-aminoimipramine-derived affinity resin. Affinity resins based on 10-hydroxyimipramine and 3-carboxylimipramine performed badly. Desipramine, immobilized via the aminomethyl group of the S-(γ-methylamino propyl) side chain, was capable of binding the 5HT-reuptake system. However, once adsorbed, the binding activity could not be recovered even under the rather stringent condition of low pH. Generally, the citalopram-derived resins showed selective retention of binding activity. In view of the moderate affinity of AMC, Ki = 460 nM, this may appear surprising. However, the low affinity of AMC as compared with citalopram might reflect a charge rather than a steric effect. The Ki values of the derivatized citalopram containing an amide bond at the 5-position of the phthalamic moiety was almost 50 times lower than the Ki value of AMC, indicating that the positively charged atom at the 5-position has a deleterious effect on the affinity for the IBS. Of the citalopram-derived resins tested, AMC-Affi-Gel 10 appeared to be the most encouraging. It exhibited biospecific retention of the 5HT-reuptake system which could be inhibited by blocking of the transporter with citalopram.

A number of chromatographic techniques, including hydrophobic interaction chromatography, chromatofocusing, and dye, lectin, and heparin chromatographies, were screened. WGA chromatography offered the most promising results, yielding a highly specific purification, combined with a high recovery and reproducability. The binding of the transporter to WGA–Con–A–Sepharose establishes the glycoproteinic nature of the 5HT-reuptake system. A serious limitation of WGA chromatography as a prefractination step is the low capacity of the resin when starting with a membrane solubilize. A potential way to overcome this handicap involves the use of a DEAE ion-exchange chromatography step prior to WGA chromatography. Application of membrane solubilize, dialyzed against 10 mM KP, buffer containing 10 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 0.3 mM DTT, and 0.1 mM PMSF, to a DE-52 cellulose column followed by an elution step using a gradient from 10 to 300 mM NaCl in the above buffer containing 0.1% digitonin gave a satisfactory 64 ± 7% recovery of binding activity at 50-100 mM NaCl (n = 6). However, the overall yield of the three-step purification procedure involving DEAE-, WGA-, and AMC-Affi-Gel chromatography was too low to allow recovery of significant binding activity in the last step.

The rationale for the elution procedure of the AMC affinity column is as follows. The column was washed with loading buffer followed by the same buffer containing 20% ethylene glycol to elute nonspecifically bound hydrophobic proteins. To achieve elution of the IBS, a buffer was used containing 10 nM citalopram but lacking NaCl. As established, the absence of NaCl reduces the affinity of the IBS for imipramine and its analogues. However, if this elution buffer was used directly after the ethylene glycol containing buffer, a considerable amount of contaminating protein eluted along with the imipramine binding activity. To avoid this, a third elution step was introduced—a buffer identical with the specific elution buffer but lacking citalopram. Application of affinity chromatography to the prepurified solubilize resulted in an overall purification of 10000-fold at a recovery of 22%. The degree of purification was calculated from the receptor density (Bmax) and a rough estimate of the protein content of the partially purified fraction from the SDS gel by using the density of the 125I-labeled BSA band as an internal standard. The relatively low recovery of the affinity step might originate from a reduced stability or from proteolytic degradation of the protein under partially purified conditions. The effect of γ-globulin, 0.3 mg/mL, and phospholipids, egg yolk l-α-phosphatidylcholine type IX-E (0.3 mg/mL), on the stability during dialysis and the AMC–Affi-Gel chromatography step was examined. No increased stability was observed.

Various studies have speculated on a reuptake system consisting of distinct, mutually exclusive, regulatory and carrier sites within the protein (Sette et al., 1983; Reith et al., 1984; Mellerup et al., 1983; Biessen et al., 1988). Viewed in this light, an identification of both the carrier site and the IBS within the partially purified preparation will be required. An appropriate way to verify the intactness of the partially purified protein involves the comparison of its pharmacological binding profile with that of the membrane-bound 5HT-reuptake system. The binding profile of the partially purified IBS shows a significant correlation (0.972 and 0.982, respectively) with that of the solubilize and of the membrane-bound protein. This observation indicates that either a copurification of a regulatory site and the carrier site has been accomplished or that both sites implicate the same protein.

The labeling intensity of both the 78- and 55-kDa proteins on SDS-PAGE corresponded well with the intensity expected on the basis of the binding activity. In some experiments, specific elution of only the 78-kDa band was observed, suggesting that the 55-kDa protein might arise from proteolytic degradation.

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Inter-Sulfhydryl Distances in Plasma Fibronectin Determined by Fluorescence Energy Transfer: Effect of Environmental Factors†

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ABSTRACT: Human plasma fibronectin, a dimeric glycoprotein, contains two cryptic free sulfhydryl groups per chain. Recent observations revealed that upon binding to a gelatin-coated surface the SH1 site, located between the DNA-binding and cell-binding domains, is partially exposed, while the SH2 site, situated within the carboxyl-terminal fibrin-binding domain, remains buried. Utilizing this newly discovered property of plasma fibronectin, we have developed a procedure to introduce maleimide derivatives of fluorescent probes such as N-(1-pyrenyl)maleimide, 7-(diethylamino)-3-(4'-maleimidylphenyl)-4-methylcoumarin, or fluorescein 5-maleimide selectively into either the SH1 or SH2 site of the fibronectin molecule and have measured the inter-sulfhydryl distances in fibronectin by fluorescence energy transfer methods. The results show that the distance between the SH1 site of one subunit and the SH1 site of the other subunit is between 35 and 44 Å, indicating the close proximity of the two subunits near the SH1-containing regions. On the other hand, the distance between the SH2 site of one subunit and the SH2 site of the other subunit is found to be greater than 95 Å, suggesting that the two SH2-containing regions are well separated. Additionally, the distance between the SH1 and SH2 sites within each subunit is estimated to be 42–53 Å, assuming no intersubunit energy transfer between the probes. Heparin or high salt, which drastically affects the hydrodynamic properties of fibronectin, had virtually no effect on the distance between the SH1–SH1 or the SH1–SH2 pair. In contrast, upon adsorption of the protein to Cytodex microcarriers, the energy transfer between the SH1 sites was markedly reduced, implying a surface-mediated separation of the two subunits of fibronectin.

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Fibronectin (Fn) is a large glycoprotein present in blood plasma and other body fluids and in tissues. It plays a part in numerous biological phenomena including cell adhesion and spreading, wound healing, phagocytosis, and differentiation [see Akiyama and Yamada (1987), McDonagh (1985), and Mosher (1984) for reviews]. The protein contains two nearly identical subunits of 240–250 kDa each, linked near their carboxyl termini by two disulfides. Within each subunit are several distinct structural domains that can be isolated from proteolytic digests with retention of specific binding affinities toward a host of macromolecules including fibrin, heparin, collagen, DNA, and cell surface molecules. Relatively little is known concerning the spatial arrangement of these structural domains of this complex protein.

Fn contains two free sulfhydryl groups per subunit: one located in a type III homology region between the DNA-binding and cell-binding domains (designated SH1) (Sorkin et al., 1986) and the other also located in a type III homology region but within the carboxyl-terminal fibrin-binding domain (designated SH2) (Garcia-Pardo et al., 1985). Neither of these sulfhydryl groups is accessible to sulfhydryl reagents in the absence of chaotropic agents (Smith et al., 1982; Lai & Tooney, 1984). Recently, we have shown that upon adsorption of Fn to a gelatin-coated surface the SH1 site is partially exposed, while the SH2 site remains buried (Narasimhan & Lai, 1989b). Here we have used this new observation to develop a procedure that allows the differential labeling of either SH1 or SH2 in Fn with maleimide derivatives of fluorescent probes (see Figure 1) and have measured inter-sulfhydryl distances of Fn by fluorescence energy transfer methods (Lakowicz, 1983; Stryer, 1978).

Materials and Methods

Fn was purified from fresh-frozen human plasma, obtained from the Blood Center of Southeastern Wisconsin, on a gelatin-Sepharose 4B affinity column (Engvall & Ruoslahti, 1977). The integrity and purity of the protein, before and after labeling with the sulfhydryl reagents, were routinely examined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The fluorescence probes N-(1-pyrenyl)maleimide (P), 7-(diethylamino)-3-(4'-maleimidylphenyl)-4-methylcoumarin (C), and fluorescein 5-maleimide (F) were purchased from Molecular Probes (Eugene, OR). Polystyrene latex beads with surface carboxyl groups were purchased from Polysciences (Warrington, PA). The beads used in this study had a diameter of 0.5 μm. Tris(hydroxymethyl)aminomethane (Tris), phenylmethylsulfonyl fluoride (PMSF), 1-[3-(diaethylamino)propyl]-3-ethylcarbodiimide hydrochloride (carbodiimide), trypsin (bovine pancreas), and heparin (porcine...