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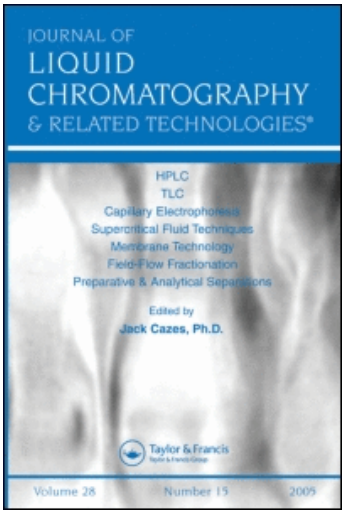

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USE OF CAPILLARY ZONE ELECTROPHORESIS FOR THE ANALYSIS OF DNA-BINDING TO A PEPTIDE DERIVED FROM AMYLOID P COMPONENT

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ABSTRACT

Capillary zone electrophoresis was used to characterize the binding interactions between oligonucleotides and synthetic peptides derived from human serum amyloid P component. From a solution containing free and peptide-bound oligonucleotides, free nucleotide was separated from the complex by means of electrophoresis. In this way, both qualitative and quantitative aspects of the binding could be assessed rapidly using minute amounts of unlabelled samples. Minimal structures and sequence specificity of binding of nucleotides and peptides could be characterized and, based on the quantitative output of the electrophoretic analysis, binding constants were estimated.

In theory, the approach is applicable for any molecular interaction where the charge/mass ratio of complexes differ from the free molecules and where at least one of the interacting components is quantitatively recoverable and detectable in the capillary electrophoresis system. As such, it is the only method available for simple and fast estimates of binding parameters for unlabelled low molecular weight compounds in solution.

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INTRODUCTION

The idea of using capillary electrophoresis (CE) to study the binding of synthetic peptides to low molecular weight ligands has recently been introduced in several publications (1-3). Additional studies illustrating the use of CE for the characterization of other types of ligand-acceptor systems have also appeared (cf. Table 1). Even though different approaches have been presented, using immobilized or free ligands and buffer or gel-filled capillaries, the basic principle, i.e., an altered electrophoretic mobility of complexed as compared to free ligand, is the same. Because of its high resolving power, speed, and quantitative abilities, CE is ideally suited for binding experiments dealing with interactions between small, unlabelled molecules where traditional approaches such as equilibrium dialysis or gel filtration are insufficient.

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The use of capillary zone electrophoresis (CZE) for characterizing the binding of anionic carbohydrates such as phosphorylated monosaccharides and heparin to synthetic peptides derived from human serum amyloid P component (SAP) has been described in a previous publication (3). As with the parent SAP protein (4), the peptide binds DNA in addition to sulfated polysaccharides (5). Here we describe the use of CZE for the qualitative and quantitative

TABLE 1

Examples of the Use of Capillary Electrophoresis for Binding Studies.

Class of Molecules Studied	Ligand	Reference
Oligodeoxynucleotides	Adenine	(9)
DNA restriction fragments	Ethidium bromide	(10)
Leucovorin stereoisomers	Bovine Serum Albumin	(11)
ß-galactose-specific lectins	Lactobionic acid	(12)
Metal-binding proteins	Ca^{++}, Zn^{++}	(13)
Synthetic peptides	Vancomycin	(1)
Synthetic peptides	Anionic carbohydrates	(3)
Carbonic anhydrase	Sulfonamides	(2)
Antigen (human growth hormone)	Antibody	(14)

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characterization of the interactions of this SAP-derived peptide, EKPLQNFTLCFR, with DNA.

MATERIALS AND METHODS

Chemicals. α-D(+)-Mannose 6-phosphate (disodium salt), 2'-deoxyadenosine 5'-monophosphate (d-AMP), and L-tyrosine were from Sigma (St. Louis, MO). Aldrich (Milwaukee, WI)

TABLE 2

Peptides Used in this Study.

Name	Sequence
Regular SAP-1 dimer	$(EKPLQNFTLCFR\text{-}NH_2)_2$
Scrambled SAP-1 dimer	$(TRLFPKECLNQF\text{-}NH_2)_2$
S-pyridylated SAP-1	EKPLQNFTLCFR-NH_2 ▌ pyridyl
S-pyridylated scrambled SAP-1	TRLFPKECLNQF-NH_2 ▌ pyridyl

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provided 2,2'-dithiodipyridine. Chemicals for HPLC and sodium tetraborate, decahydrate came from J.T. Baker (Phillipsburg, NJ), boric acid and hydrogen peroxide from Fisher Scientific (Fair Lawn, NJ). Oligonucleotides (Oligodeoxyadenylic acids: $(pdA)_2$ and $(pdA)_6$, sodium salts) were from Pharmacia LKB (Piscataway, NJ). All chemicals for peptide synthesis were purchased from Applied Biosystems, Inc. (Foster City, CA).

Peptide Synthesis. Peptides (cf. Table 2) were synthesized on an Applied Biosystems 430A automated solid-phase synthesizer using t-BOC chemistry and deprotected and purified as previously described (3). The composition of

the peptides were verified by amino acid analysis. The cysteine-containing peptides were protected against oxidative degradation by dimerization through oxidative formation of disulfide-linked homodimers or S-pyridylation of peptide monomers as previously described (3).

Electrophoresis. Electrophoresis was performed in 72 cm long untreated fused-silica capillaries of 50 μm in diameter (Applied Biosystems) with the detector at 50 cm using an Applied Biosystems 270A capillary electrophoresis system connected to a Spectra-Physics SP4400 integrator. Between electrophoretic runs, the capillary was washed in 0.1 M NaOH for one min and then in electrophoresis buffer for 3 min. Electrophoresis took place at 30 kV (anode at the sample injection end of the capillary), the temperature was set at 30°C, and detection was at 200 nm (range: 0.004; risetime: 0.05). Samples were introduced by vacuum injection (2 s injection corresponds to ca. 7 nl sample). Electrophoresis buffer was either 20 mM sodium citrate, pH 2.5 (Applied Biosystems) or a borate buffer, pH 7.3 (18 mM boric acid, 0.5 mM sodium tetraborate, 4.6 mM sodium chloride) resulting in currents of ≈ 19 μA and ≈ 8 μA, respectively.

Binding Assays. For binding studies involving mannose 6-phosphate, the peptides at final concentrations from 26 μM to 3.9 mM were incubated in dilute TBS (0.05 mM Tris/HCl,

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pH 7.4, 1.5 mM NaCl) with 3.5 mM mannose 6-phosphate in a volume of 110 μl in polypropylene tubes. After overnight incubation at ambient temperature, each incubation was divided into three separate tubes and was analyzed by CZE using 2.5 s vacuum injections and 20 mM citrate, pH 2.5 as the electrophoresis buffer. Integrated peak areas were adjusted for small differences in migration times and the amount of free peptide represented by these peak areas was then calculated using standard curves of peak areas versus amount of peptide. Knowing the total amount of peptide present in each incubation, binding curves of bound versus total could then be obtained (3).

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For the nucleotide studies, all incubations were performed in the electrophoresis buffer (borate, pH 7.3, see above). Stock solutions of dAMP and oligonucleotides were 0.78 mM in HPLC-grade water and kept frozen at -20°C.

To examine the minimal structural requirement for nucleotide binding to the peptide, nucleotides of different sizes at a final concentration of 67 μM were mixed with peptides (scrambled or regular) at a final concentration of 152 μM in a volume of 22 μl borate, pH 7.3 containing 13 μg/ml tyrosine as a marker. These mixtures were then analyzed by CZE using borate, pH 7.3 as the electrophoresis buffer and 2 s (hexanucleotide) or 6 s (mono- and dinucleotides) vacuum injection.

The specificity of the interaction of the peptides with DNA was examined in experiments where a constant

concentration of oligonucleotide (67 μM) was incubated with a constant concentration of peptides (152 μM) composed of various ratios of scrambled to regular sequence peptide in a volume of 22 μl. After incubation, these samples were centrifuged for 5 min at 12,000 x g_{av} before being analyzed by CZE because the incubations with the peptide dimer resulted in precipitate formation.

For the binding studies with $(pdA)_6$, a constant amount of peptide (90 μM) was incubated in triplicate overnight at ambient temperature with varying amounts (7 to 85 μM) of the oligonucleotide in a final volume of 17 μl which also contained 22 μg/ml of tyrosine as a marker. CZE was then performed using 2 to 6 s vacuum injections. After migration time-based area adjustments, binding curves for the oligonucleotide could be constructed as outlined above. The nucleotides were assigned the following molecular weights: dAMP, 331.2; $(pdA)_2$, 641.4; $(pdA)_6$, 1890.2).

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RESULTS AND DISCUSSION

The synthetic SAP-1 peptide (cf. Table 2) previously was shown by CZE and other methods to bind heparin (3) and other sulfated polysaccharides (Batinić et al., unpublished results). In addition to the interaction with these compounds, one of the prominent characteristics of the parent protein, human serum amyloid P component (SAP), is

its binding to certain phosphorylated monosaccharides and DNA (4,6). The present study was undertaken to examine if these binding activities were also mimicked by the synthetic peptide. In a previous paper (3), we had demonstrated the usefulness of CZE for these analyses. The amount of free peptide after incubation with anionic ligands was estimated after a charge-dependent electrophoretic separation of complexed from free peptide using a pH of 2.5 for the separation.

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The same approach was employed to see if the SAP-1 peptide interacted with mannose 6-phosphate as has been reported to be the case for the parent protein (6). By use of CZE, a decrease in the amount of free peptide proportionally with the presence of increasing amounts of the mannose 6-phosphate during the preincubation could be demonstrated.

About 4 times less peptide appeared to be bound when using glucose 6-phosphate as the ligand under identical conditions (not shown). The structural demands of the peptide for this interaction was examined by letting a regular and a scrambled version of the SAP-1 peptide (cf. Table 2) interact with mannose 6-phosphate. The graph of the results which is shown in Figure 1 illustrates that there is a considerable difference in binding to the mannose 6-phosphate between these two versions of the peptide. The scrambled peptide binds less than SAP-1 itself and, therefore, the interaction depends on a

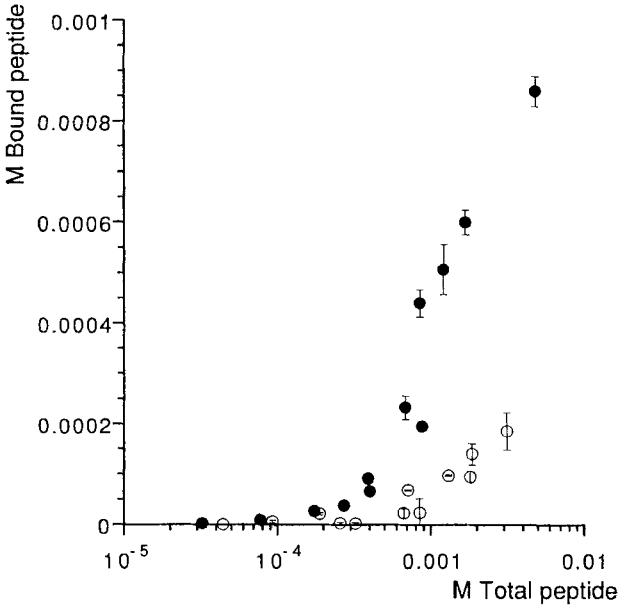

FIGURE 1. Specificity of the interaction of SAP-1 with a phosphorylated monosaccharide. CZE-derived binding curves for the interaction of mannose 6-phosphate with the S-pyridylated SAP-1 (●) and the S-pyridylated scrambled SAP-1 (○). Various concentrations of peptides were incubated with 3.5 mM mannose 6-phosphate in dilute TBS. The mixtures were subsequently analyzed by CZE as described in MATERIALS AND METHODS using 20 mM sodium citrate, pH 2.5 as the electrophoresis buffer. The amount of bound peptide was then calculated from the integrated peak areas which were taken as a measure of free peptide.

specific amino acid sequence and is not a reflection of a charge-dependent electrostatic interaction amplified by the low ionic strength of the buffer.

The above-mentioned experiments seemed to confirm specific interactions of SAP-1 with certain phosphorylated monosaccharides which resembled the binding pattern of the

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parent protein. However, the conditions for incubation and electrophoresis were not physiological. The peptides are quite hydrophobic and it was necessary that the incubation buffer be dilute TBS in order for the peptides to remain in solution. Furthermore, to recover the peptides in CZE, the electrophoresis was performed at pH 2.5 which minimized interactions with the capillary wall.

It therefore was desirable to design conditions for binding experiments at neutral pH using a higher ionic strength. Since the peptides were not recoverable at neutral pH, it was necessary to use the oligonucleotides as the detected analyte in these assays. Figure 2 illustrates this approach where deoxy-nucleotides of different sizes are analyzed at pH 7.3 after incubation with scrambled or regular SAP-1 dimer in the electrophoresis buffer (borate, pH 7.3). Under these near-native conditions, nucleotide binding to the peptide prior to electrophoresis is unperturbed by the processes of sample injection and subsequent electrophoresis and the peptide-bound nucleotide is then retarded by peptide-wall interactions. Figure 2 illustrates the specificity of peptide binding and the minimum nucleotide structure required for this binding. Only SAP-1 affects the oligonucleotides and a maximum of six nucleotides are required for binding. Experiments like these also were performed to estimate the minimum peptide-sequence required for binding. It was found that it was

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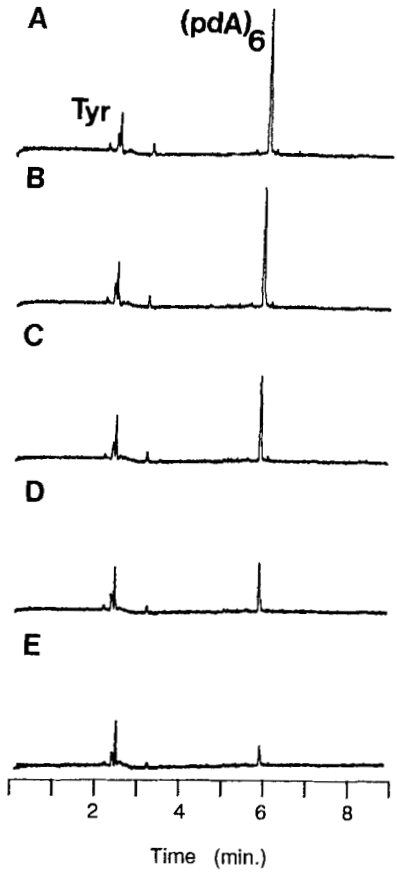

FIGURE 2. Minimal nucleotide requirement and specificity of peptide binding. CZE analysis of mono- (A and B), di- (C and D), and hexa-deoxyadenylic acid (E and F). The nucleotides were incubated at 67 μM with 152 μM scrambled (left column) or regular SAP-1 dimer (right column) in borate, pH 7.3. Tyrosine (TYR) was included as a marker at 13 μg/ml. Analysis was after 2-6 s vacuum injections of supernatants after 5 min centrifugation at 12,000 x g_{av}.

possible to truncate the sequence down to FTLCFR-NH_2 without appreciably affecting the binding (not shown).

Having established this CZE-based screening method for peptide-nucleotide interactions at a neutral pH, the approach was applied to a binding study of the interaction between the 6-mer oligodeoxyadenylic acid $(pdA)_6$ and the SAP-1 peptides. First, it was established that there was a proportionality between amount of added peptide and detected oligonucleotide by performing the experiment shown in Figure 3 in which constant amounts of oligonucleotide and peptides were co-incubated. The peptide was a mixture of regular and scrambled SAP-1 dimer in different proportions, and these experiments clearly illustrated a linear relationship between the amount of the regular peptide and the amount of detected, free oligonucleotide. Thus, the interactions were specific and quantifiable.

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The assay was then used to construct the binding curves of Figure 4 which are based on experiments in which different quantities of DNA were incubated with a constant amount of the S-pyridylated SAP-1 monomer. The curvilinear Scatchard plot (Figure 4B) suggests the presence of different classes of binding sites and/or cooperative effects (7). It could be hypothesized that the binding of additional peptide monomers to the 6-mer oligonucleotide is counteracted by steric repulsion. On the basis of the binding curve an average dissociation constant (corresponding to half-saturation) was estimated to be ca.

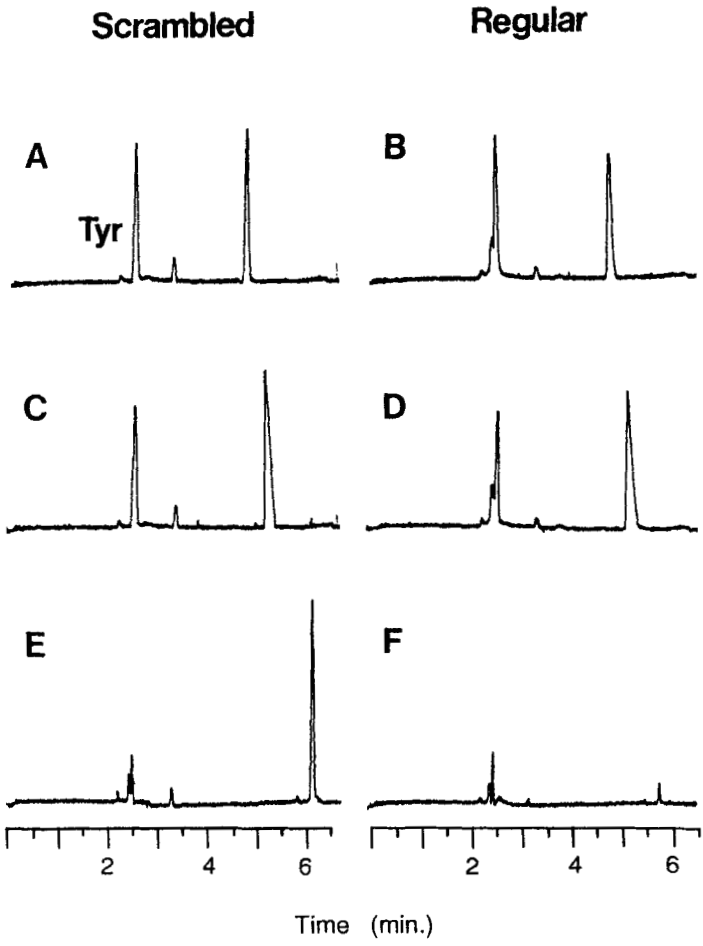

FIGURE 3. Specificity of the interaction of SAP-1 with the $(pdA)_6$ oligonucleotide. CZE analysis of incubations in borate, pH 7.3 of 67 μM $(pdA)_6$ with different ratios of scrambled to regular SAP-1 dimer giving a constant peptide concentration of 152 μM; A, 20 %; B, 40 %; C, 60 %; D, 80 %; and E, 100 % regular SAP-1 dimer. Samples analyzed after 5 min centrifugation at 12,000 x g_{av}. Tyrosine (TYR) at 13 μg/ml was included as an internal reference.

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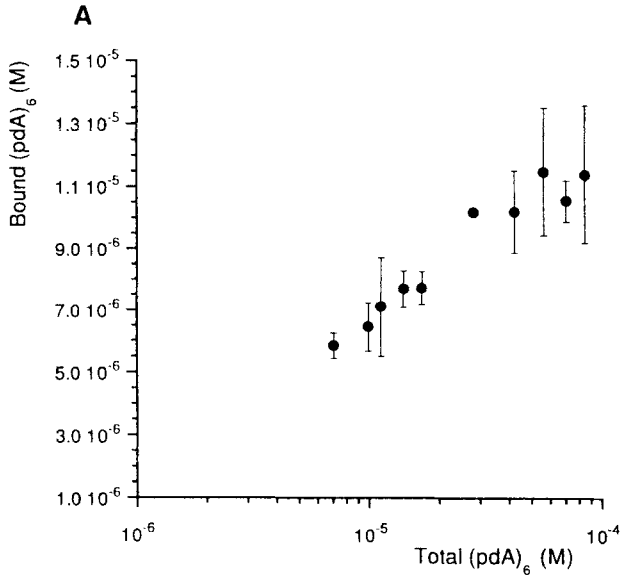

FIGURE 4. Interaction of S-pyridylated SAP-1 monomer with $(pdA)_6$ oligonucleotide. CZE-derived binding curves. A, Graph of experimental binding data; B, Scatchard plot of the same data. A constant concentration of peptide (90 μM) was incubated with various concentrations (7-85 μM) of $(pdA)_6$ and subsequently analyzed by CZE in borate, pH 7.3. Results are depicted as the means $\pm$ the standard deviations of the triplicate incubations. A visual decomposition of the Scatchard plot yields dissociation constants of 0.5 to 5 μM.

4 μM. A visual decomposition of the Scatchard plot into two straight lines gave dissociation constants of 0.5 and 5 μM.

Conclusively, CZE has proven useful for the detection and characterization of peptide-DNA interaction under mild conditions. The fine selectivity of the parent protein, SAP, for different anionic carbohydrates also has been

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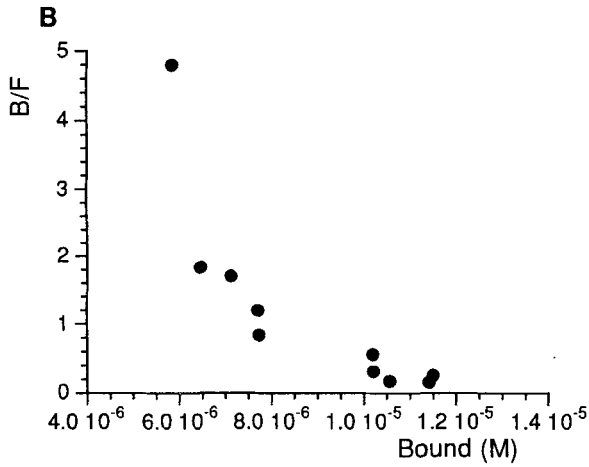

FIGURE 4 (continued)

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demonstrated by the synthetic peptides derived from the parent SAP and the specificity and strength of these interactions were shown by CZE.

For studies of interacting low molecular weight molecules, this might be the only simple approach to take when working with unlabelled molecules in solution. As shown here, the assay may be used to determine binding constants simply by making it possible to quantitate unbound material after establishment of equilibrium. This is feasible if at least one of the components participating in the interaction can be recovered quantitatively under the conditions of the electrophoresis. Non-equilibrium methods where the ligand is distributed homogeneously throughout the capillary prior to electrophoresis and where

binding constants are calculated on the basis of changes in migration times (1,2) are more dependent on suitable on-off rates (i.e. fast as compared to the time for the electrophoresis (8)). The simple CZE-based quantitation of bound and free ligand/acceptor after establishment of equilibrium as in this study only assumes that the processes of sample injection and start of electrophoresis do not disturb the binding.

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