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Capillary Electrophoresis and Fluorescence Anisotropy for Quantitative Analysis of Peptide−Protein Interactions Using JAK2 and SH2-Bβ as a Model System

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Fluorescence anisotropy capillary electrophoresis (FACE) and affinity probe capillary electrophoresis (APCE) with laser-induced fluorescence detection were evaluated for analysis of peptide−protein interactions with rapid binding kinetics. The *Src* homology 2 domain of protein SH2-Bβ (SH2-Bβ (525−670)) and a tyrosine-phosphorylated peptide corresponding to the binding sequence of JAK2 were used as a model system. For peptide labeled with fluorescein, the K_d = 82 ± 7 nM as measured by fluorescence anisotropy (FA). APCE assays had a limit of detection (LOD) of 100 nM or 12 amol injected for SH2-Bβ (525−670). The separation time of 4 s, achieved using an electric field of 2860 V/cm on 7-cm-long capillaries, was on the same time scale as complex dissociation allowing K_d (101 ± 12 nM in good agreement with FA measurements) and dissociation rate (k_{off} = 0.95 ± 0.02 s^{-1} corresponding to a half-life of 0.73 s) to be determined. This measurement represents a 30-fold higher rate of complex dissociation than what had previously been measurable by nonequilibrium CE analysis of equilibrium mixtures. Using FACE, the protein was detected with an LOD of 300 nM or 7.5 fmol injected. FACE was not used for determining K_d or k_{off}; however, this method provided better separation resolution for multiple forms of the protein than APCE. Both methods were found suitable for analysis of cell lysate. These results demonstrate that FACE and APCE may be useful complements to existing techniques for exploring binding interactions with rapid kinetics.

Cellular chemistry is controlled by affinity interactions between biomolecules. Quantitative analysis of such interactions is important in developing an understanding of how reactions are organized within cells and for developing drugs or chemical probes of cellular processes. A variety of affinity methods based on capillary electrophoresis (CE) have emerged as potentially useful instrumental approaches for quantitative analysis of noncovalent interactions between biomolecules.[1,2] The objective of this work was to extend the utility of CE methods to binding systems that have rapid on−off kinetics. As many cellular chemistry interactions rely on such rapid binding, these improved methods are expected to have utility in signal transduction studies.

Affinity CE methods can be classified as nonequilibrium methods, in which binding partners are mixed and then free and bound components separated by electrophoresis, or equilibrium methods, in which one of the binding partners is added to the separation buffer and the other binding partner injected and separated. In the latter case, the binding interaction is detected by a migration time shift or by a change in fluorescence property. The nonequilibrium methods include noncompetitive assays,[3] competitive assays,[4] nonequilibrium CE of equilibrium mixtures (NECEEM),[5−7] and affinity probe CE (APCE).[8] Equilibrium methods include affinity CE (ACE),[9−11] affinity CE with laser-induced fluorescence polarization detection,[12,13] and fluorescence anisotropy CE (FACE).[14] When compared to other methods such as surface plasmon resonance and fluorescence anisotropy (FA), the CE techniques offer the advantage of utilizing separations, which allows analysis in complex mixtures and analysis of multiple interactions or binding partners at one time, in binding assays. CE methods are also inherently miniaturized and therefore use

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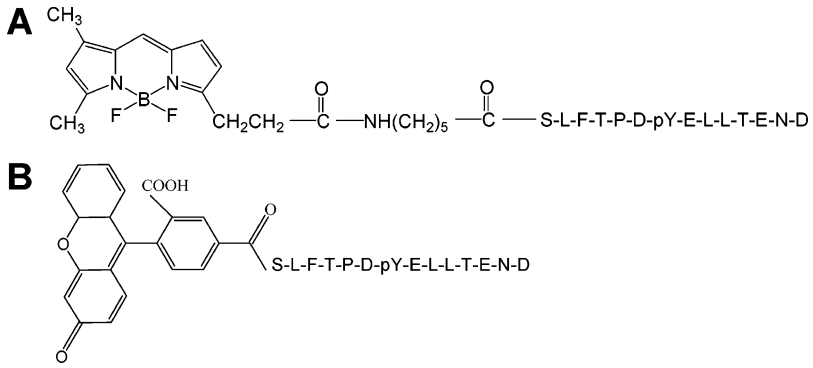

Figure 1. Structure of BODIPY-JAK2 813 peptide (A) and Fluor-JAK2 813 peptide (B).

much less sample than other techniques, which is especially important in biochemical studies where limited quantities are available.

While equilibrium CE methods have been used for rapid binding systems, nonequilibrium CE methods have only been used for binding reactions that have relatively low dissociation rates, on the order of min^{-1}, because for reactions with high dissociation rates the separation time is too long to detect complex. The development of techniques for rapid CE separations, along with mathematical tools for analyzing electrophoretic data,[5−7] has opened the possibility of using nonequilibrium methods, like APCE, for analyzing interactions with more rapid kinetics; however, this has yet to be demonstrated. In this work, we test the hypothesis that nonequilibrium separation analysis can be used for systems with more rapid dissociation rates ($>2\ s^{-1}$) when using conditions suitable for rapid CE.

The model system used in this study is a *Src* homology 2 (SH2) domain protein binding a phosphorylated peptide. SH2 domain proteins play an important role in cellular signal transduction by binding to proteins at phosphorylated tyrosine residues resulting in cascades of cellular chemistry changes.[15,16] The three residues immediately following the phosphotyrosine are a key determinant of specificity for SH2 binding; therefore, small peptides encompassing this sequence can be used for assays.[17] SH2 domain protein and phosphopeptide binding have been reported to have rapid binding kinetics with off-rates of $0.1-1\ s^{-1}$, making them a good model system for this study.[15,18]

The protein used is the SH2 domain of SH2-Bβ, an adaptor protein that binds JAK2, a member of the Janus family of cytoplasmic tyrosine kinases involved in cytokine signaling.[19−21] The SH2 domain of SH2-Bβ (amino acids 525−670) binds to JAK2 at tyrosine 813 after that site is autophosphorylated in response to growth hormone.[20] Binding experiments were performed using an oligopeptide, corresponding to the expected binding sequence of JAK2, which was labeled with BODIPY FL or 5-carboxyfluorescein (Figure 1) on the N-terminus. Binding assays were performed using FACE, APCE, and FA assays to allow comparison of the techniques. We demonstrate that although the protein−peptide complex has a half-life of less than 1 s, it can be readily detected by the CE methods. Furthermore, the first quantitative measurements of binding equilibria and kinetics are determined for the SH2-Bβ and JAK2 interaction.

EXPERIMENTAL METHODS

Chemicals. Tris−glycine buffer (10×) was purchased from Bio-Rad laboratories (Hercules, CA). All solutions were prepared with deionized water from an E-Pure water purification system (Barnstead International Co., Dubuque, IA). 5-carboxyfluorescein, succinimidyl ester (5-FAM, SE), 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)amino) hexanoic acid succinimidyl ester (BODIPY FL, SE) and rhodamine 110 were purchased from Molecular Probes (Eugene, OR). Dulbecco's Modified Eagle Medium (DMEM) was from Invitrogen (Carlsbad, CA). Aprotinin and Leupeptin were from Roche. JAK2 tyrosine 813-phosphorylated peptide with fluorescein label (Fluor-JAK2 813 peptide) and BODIPY label (BODIPY-JAK2 813 peptide) were synthesized and labeled by the Protein Core of the Michigan Diabetes Research and Training Center (see Figure 1 for structures of both peptides). SH2-Bβ (525−670) was expressed and purified as a fusion protein with glutathione *S*-transferase (GST) as previously described.[22] The GST tag was incorporated for purification by glutathione-agarose beads.[23]

COS-7 Cell Culture and Cell Lysis. A 100 mm × 20 mm cell culture dish (Fisher Scientific) of COS-7 cells were grown to confluence in DMEM supplemented with 1 mM L-glutamine, 100 units of penicillin/mL, 100 μg of streptomycin/mL, 0.25 μg of amphotericin/mL, and 8% fetal bovine serum. Cells were then washed three times in chilled phosphate-buffered saline (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na_3VO_4, pH 7.4) and solubilized in lysis buffer (50 mM Tris, pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 2 mM ethyleneglycol−bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 1 mM Na_3VO_4, pH 7.5), containing 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/mL), and leupeptin (10 μg/mL). Cell lysates (1.25×10^6 cells/mL) were centrifuged at 16750 × *g* for 10 min, and the supernatant was saved for further analysis.

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Fluorescence Anisotropy Experiments. FA measurements were performed on a Perkin-Elmer Fusion Universal Microplate Analyzer (Packard Instrument Co., Meriden, CT). Fluorescence was excited at 485 ± 10 nm and emission collected with a 535 ± 12 nm band-pass filter. The factory default G value (0.9) was used for all assays. For protein−peptide binding assays, samples were made to the desired concentration (100 nM BODIPY-JAK2 813 peptide or 20 nM Fluor-JAK2 813 peptide with 0−700 nM GST−SH2-Bβ) in 25 mM Tris, 192 mM glycine buffer at pH 8.5. FA was measured immediately after mixing. To determine nonspecific binding, 10 μM unlabeled JAK2 813 peptide was added to each of the above protein−peptide mixtures, other conditions being the same. Specific binding was determined by subtracting nonspecific binding from total binding. The dissociation constant (K_d) was determined by fitting data to

$$r = r_F \cdot \frac{K_d}{[P_0] + K_d} + r_B \frac{[P_0]}{[P_0] + K_d} \quad (1)$$

where r is the measured anisotropy, $[P_0]$ is the initial protein concentration, and r_B and r_F are anisotropy values of the complex and free ligand, respectively. We assumed r_F to be the measured anisotropy when protein concentration was zero.

FACE Assays. FACE assays were performed using an experimental setup similar to that described previously.[14] Electrophoresis was performed using a high-voltage power supply (Spellman CZE 1000 R, Plainview, NY) applying 400 V/cm across an eCAP neutral capillary (50-μm i.d., 375-μm o.d., total length 45.2 cm, length from inlet to detector 33 cm, Beckman-Coulter, Fullerton, CA). Vertically polarized 488-nm light from a Kr^+ laser was used for excitation. Fluorescence emission was collected at 90° to the excitation and spatially filtered before being split into its parallel and perpendicular components by a polarizing beam splitter cube. Each polarized component was filtered by a 520 ± 10 nm band-pass filter and detected by a PMT. Anisotropy was calculated from the signals acquired at both PMTs. Data acquisition and calculation of anisotropy were performed using a LabView (National Instruments, Austin, TX) program written in-house running on a personal computer equipped with a National Instruments AT-MIO-16 data acquisition card.

Electrophoresis buffers contained 25 mM Tris, 192 mM glycine, and the desired concentration of peptide at pH 8.5. Samples containing purified GST−SH2-Bβ (525−670) or cell lysate and Fluor- or BODIPY-JAK2 813 peptide were premixed before separation. All dilutions were made in Tris−glycine buffer. For sample loading, the capillary inlet was elevated to a height of 10 cm for 20 s to allow sample to be injected by gravity. At the beginning of each day, the separation capillary (eCAP neutral capillary) was rinsed with 5% acetic acid, water, and separation buffer for 5 min each.

APCE-LIF Assays. The flow-gated CE-LIF instrument used for APCE assays was described previously.[24] An unmodified fused-silica capillary (10-μm i.d., 360-μm o.d., total length 7 cm, inlet to detector length 3.5 cm) was used as the separation capillary. All samples were introduced onto the capillary by electrokinetic injections via a flow gate interface[25] at 2 kV for 0.2 s and separated at 20 kV except for APCE-LIF assays with BODIPY-JAK2 813 peptide in which 15 kV was applied. Tris−glycine buffer was continuously delivered to the flow gate at a flow rate of 1.0 mL/min by a Series I HPLC pump (LabAlliance, Fisher Scientific, Pittsburgh, PA).

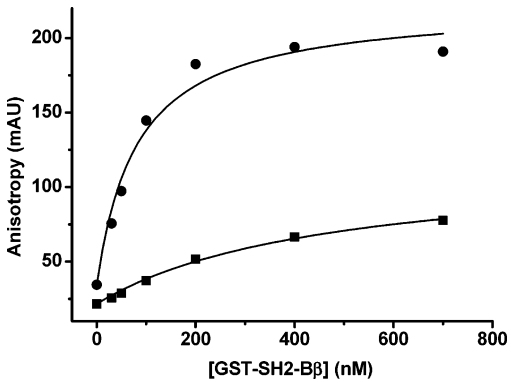

Figure 2. FA measurements for binding of GST−SH2-Bβ (525−670) and Fluor-JAK2 813 peptide (●) or BODIPY-JAK2 813 peptide (■). Samples contained 20 nM Fluor-JAK2 813 peptide or 100 nM BODIPY-JAK2 813 peptide and 0−700 nM GST−SH2-Bβ (525−670) in Tris−glycine buffer pH 8.5.

Samples containing Fluor- or BODIPY-JAK2 813 peptide and GST−SH2-Bβ (525−670) were made to the desired concentration in Tris-glycine buffer and immediately assayed using flow-gated CE-LIF. The 10 nM rhodamine 110 was added to all samples as internal standard.

The K_d was determined by

$$K_d = [P]_0 \frac{A1}{A2 + A3} - [L]_0 \frac{A1}{A1 + A2 + A3} \quad (2)$$

where $[L]_0$ is the initial ligand concentration and $A1$, $A2$, and $A3$ are areas of free peptide peak, complex peak, and exponential part of the electropherogram, respectively.[5] The dissociation rate constant (k_{off}) was determined using

$$k_{off} = -\frac{\ln\left(\frac{A2}{A2 + A3}\right)}{t_c} \quad (3)$$

where t_c is the migration time of the complex.[7] Half-life was calculated from $t_{1/2} = \ln 2/k_{off}$.

RESULTS AND DISCUSSION

Anisotropy Measurements. Initial experiments were aimed at using FA to characterize binding of the fluorescent JAK2 813 peptides and GST−SH2B-β (525−670). Figure 2 compares the effect of protein concentration on FA of the two peptides. (No significant fluorescence intensity change was observed upon binding.) The K_ds for fluorescein and BODIPY-labeled peptide were determined from these curves to be 82 ± 7 (n = 3) and 467 ± 10 nM (n = 3), respectively, using eq 1. Thus, the BODIPY-labeled peptide has lower affinity and a lower net anisotropy change than the fluorescein-labeled peptide (compare anisotropy

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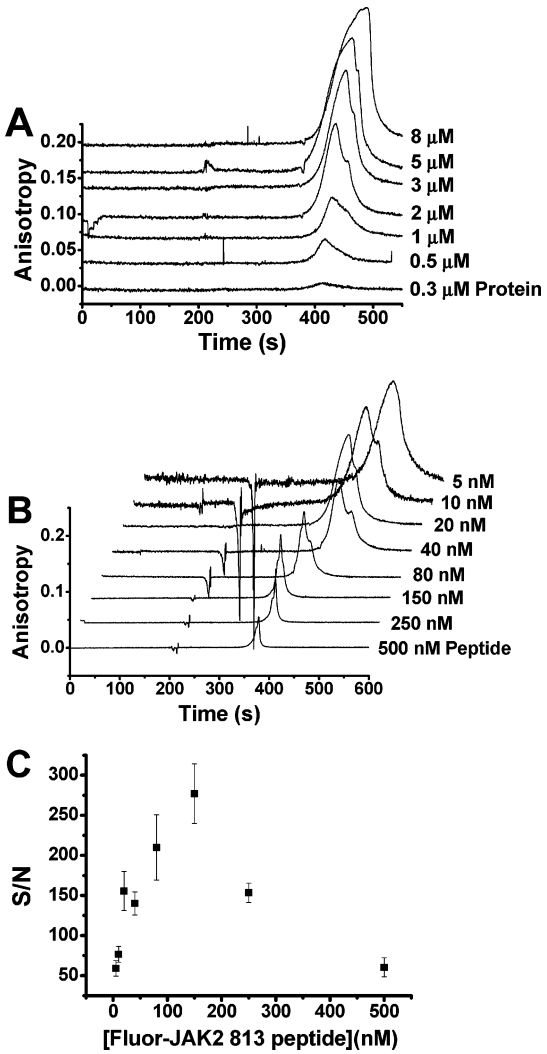

Figure 3. FACE assays of GST−SH2-Bβ (525−670). (A) Electrophoresis buffer contained 20 nM Fluor-JAK2 813 peptide. [GST−SH2-Bβ (525−670)] was increased from 0.3 to 8 μM. (B) Sample contained 2 μM GST−SH2-Bβ (525−670). Electrophoresis buffer contained Fluor-JAK2 813 peptide with concentration varied from 5 to 500 nM. Separation conditions of (A) and (B) were as described in the Experimental Section under FACE Affinity Assays. (C) Signal-to-noise ratio (S/N) at peptide concentration from 5 to 500 nM corresponding to electropherograms in (B).

at saturation in Figure 2). The BODIPY construct has a longer spacer between fluorophore and peptide, which could possibly lead to more rotational freedom and lower anisotropy of the fluorophore even when a complex is formed. Given these results, Fluor-JAK2 813 peptide was used for the majority of the FACE and APCE-LIF assays.

FACE Assays. We have recently introduced FACE as an equilibrium affinity CE method in which unlabeled proteins are separated by CE using buffer that contains a fluorescent binding partner (peptide in this case) and fluorescence anisotropy is monitored at the detection point.[14] As unlabeled protein migrates through the detection zone, it increases the anisotropy signal if it binds the fluorophore. This technique allows, in principle, mixtures to be separated and detected based upon their binding to a fluorescent ligand. Figure 3A illustrates FACE assay results for samples that contained 0.3−8 μM GST−SH2-Bβ (525−670) with 20 nM Fluor-JAK2 813 peptide in the electrophoresis buffer. The positive anisotropy feature that increases with increasing protein concentration results from detection of a protein−peptide complex that has high anisotropy compared to background peptide. With 20 nM peptide in the electrophoresis buffer, the limit of detection (LOD) for GST−SH2-Bβ (525−670) was 300 nM. The precision of individual assays depended upon protein concentration so that at 8 μM protein the relative standard deviation (RSD) of multiple measurements was 0.3% ($n = 8$) but at 300 nM the RSD was 19% ($n = 8$).

The concentration of fluorescent peptide in the electrophoresis buffer had large effects on both peak shape and S/N for FACE assays. Figure 3B shows that the complex peak tends to narrow with increasing peptide concentration. This effect on zone broadening has previously been observed with ACE measurements and is due to the effect of equilibration of the protein between bound and unbound states, each of which migrates at a different rate.[26] Although the peak narrowed with higher concentration, resolution actually appeared better at moderate peptide concentrations. The presence of multiple peaks was surprising given that the protein was purified for these experiments. This result suggests that multiple forms of the protein are produced in the cells, perhaps due to different conformations or posttranslational modifications of the protein. It is also possible that the protein degrades after purification.

Figure 3B also illustrates that the anisotropy signal tended to decrease with increasing peptide signal, consistent with the theoretical model established previously.[14] Because anisotropy is linearly additive, the contribution of bound and free fluorescent peptide to total anisotropy is determined by both the fraction bound and the anisotropy of each species. As peptide concentration increases, the amount of complex formed increases; however, the fraction bound is less and the net anisotropy tends to approach that of the free species since a larger proportion of the anisotropy is due to the free species. Although the signal increases with lower peptide concentration in electrophoresis buffer, noise in the anisotropy trace increased as fluorophore concentration decreased. Because of these competing effects, the S/N had an optimum as illustrated in Figure 3C. We estimate the detection limit for the protein at the optimal peptide concentration to be 100 nM. (Detection limit was determined as the concentration that would yield S/N of 3 where the noise was the root mean square of the baseline signal.) The effect of noise at low concentrations was not considered in the previous model of FACE.[14]

To demonstrate the capability of FACE for assays in complex mixtures, lysates from *Escherichia coli* cells overexpressing GST−SH2-Bβ (525−670) and unmodified COS-7 cells were analyzed using Fluor-JAK2 813 peptide added to the electrophoresis buffer (Figure 4). For *E. coli* cell lysate, the migration time of the complex was identical to that of the purified protein. The protein concentration within the lysate of *E. coli* overexpressing SH2-Bβ (525−670) was estimated from the peak height to be ~5 μM. Cell lysate contained ~1.2×10^{10} cells/mL and 25 nL was injected so the signal detected corresponds to 0.4 amol/cell (~3×10^5 cell equivalents injected). A putative SH2-Bβ peak was also detectable in lysate from COS-7 cells, a simian kidney cell line expected to naturally express this protein (Figure 4B). The migration time

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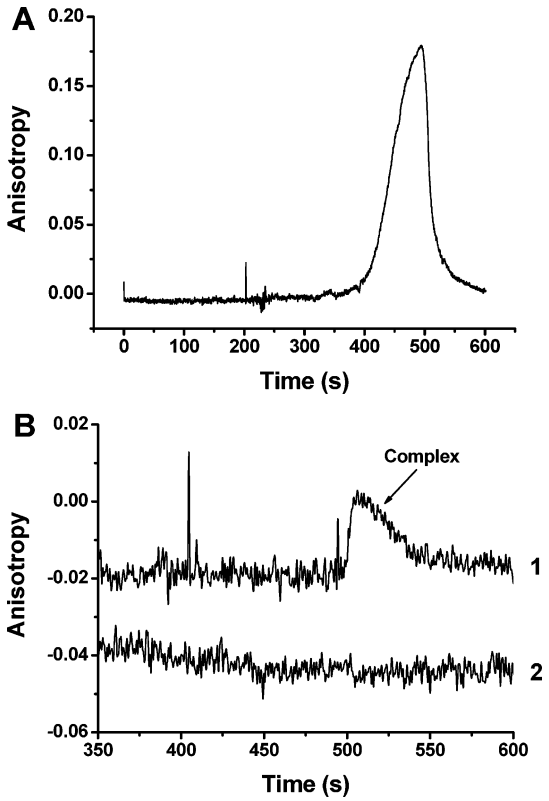

Figure 4. FACE assays of lysate from *E. coli* cells overexpressing GST−SH2-Bβ (525−670) (A) and COS-7 cells (B). (A) Electrophoresis buffer contained 20 nM Fluor-JAK2 813 peptide. (B) Electrophoresis buffer contained 20 nM Fluor-JAK2 813 peptide in the absence (trace 1) or in the presence of (trace 2) 500 nM unlabeled JAK2 813 peptide. All separation conditions were the same as in Figure 3.

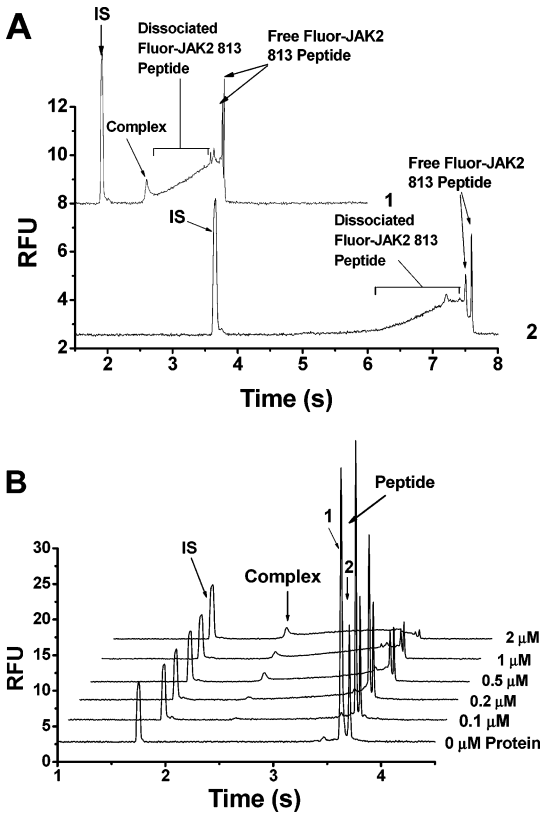

Figure 5. APCE-LIF assays of GST−SH2-Bβ (525−670). (A) Effect of electric field on complex detection. Sample contained 1 μM GST−SH2-Bβ (525−670), 100 nM Fluor-JAK2 813 peptide, and 10 nM internal standard (IS, rhodamine 110) in both traces 1 and 2. Electric field was 2860 (trace 1) and 1400 V/cm (trace 2). (B) Sample contained 100 nM Fluor-JAK2 813 peptide, 10 nM R110, and 0−2 μM GST−SH2-Bβ (525−670). Peaks 1 and 2 are due to different isomers of the Fluor-JAK2 813 peptide. Separation conditions were described as in the Experimental Section under APCE-LIF Assays.

was slightly greater than that seen with the overexpressed protein; however, the protein detected in this experiment should be full-length SH2-Bβ without a GST tag and therefore would be expected to migrate differently. The detected signal appeared to be due to specific binding to labeled peptide because it could be eliminated by adding 500 nM unlabeled peptide to the electrophoresis buffer (lower trace in Figure 4B). Using calibration with GST−SH2-Bβ (525−670), we estimated the concentration of SH2-Bβ in COS-7 cell lysate to be 300 nM corresponding to 200 amol/cell. These results demonstrate that SH2 domain proteins can be detected by FACE in complex samples. The method would also appear to be compatible with highly miniaturized analysis as the mass detection limits are low and the volume of injected COS-7 cell lysate corresponds to ~30 COS-7 cells.

APCE-LIF assays. We then examined the use of Fluor-JAK2 813 peptide as an affinity probe for detection of GST−SH2-Bβ (525−670) by APCE. Because the kinetics of binding were expected to be fast, flow-gated CE with short columns was used to achieve rapid separation. Figure 5A shows the result of an APCE assay of GST−SH2-Bβ (525−670). For this assay, 1 μM GST−SH2-Bβ (525−670) and 100 nM Fluor-JAK2 813 peptide were premixed and injected electrokinetically. In trace 1, the separation was completed in 4 s using an electric field of 2860 V/cm and peaks corresponding to complex and free peptide are detected. The complex peak is confirmed by observing that it increased with increasing protein while the free peaks decreased (Figure 5B) and was eliminated by addition of excess unlabeled peptide to the mixture (data not shown). Two peaks for peptide were detected, likely due to separation of two isomers of fluorophores. In addition to the peaks, a bridge between the peaks was detected that corresponded to peptide that dissociated from the complex during separation, an effect observed when separation occurs on the same time scale as the complex half-life.[5] At 1400 V/cm, with a separation time of 8 s, the complex peak was not detected, probably because it had completely dissociated (trace 2, Figure 5A) on this time scale. (Similar results were obtained with longer columns and longer times.) These results indicate the importance of short separation times for detection of rapidly dissociating complexes. Analysis of samples containing different concentrations of protein had RSDs of 3−5% for peptide peak heights ($n = 10$ for each sample), demonstrating good reproducibility of this method.

The presence of two active peptides could complicate quantitative analysis of binding; however, both peptide peaks decreased with increasing protein concentration to a similar degree, indicating that they bound with comparable affinity (data not shown). Therefore, we used the sum of the two peptide peak areas for the calculation of K_d and k_{off}.

Krylov's group recently demonstrated an approach, NECEEM, that uses eqs 2 and 3 to determine K_d and k_{off} from APCE data when dissociated ligand can be detected, as in Figure 5.[5,7] Figure

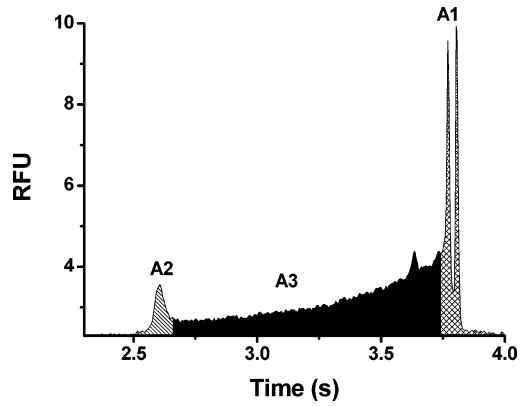

Figure 6. Sample electropherogram used in determination of K_d and k_{off} by APCE-LIF. Sample contained 0.5 μM GST−SH2-Bβ (525−670), 100 nM Fluor-JAK2 813 peptide, and 10 nM rhodamine 110. Separation conditions were the same as in Figure 5. Areas marked correspond to bound peptide (A2), peptide that dissociated during the separation (A3), and peptide that was free in the sample solution (A1). Calculations of K_d and k_{off} are described in the Experimental Section under APCE-LIF Assays.

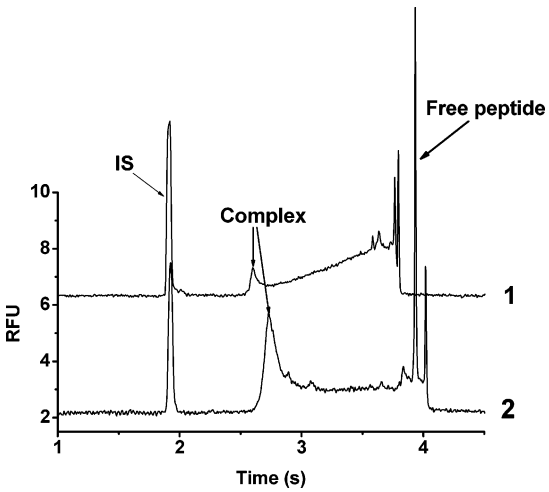

Figure 7. APCE-LIF assays for purified GST−SH2-Bβ (525−670) and cell lysate. Sample contained 100 nM Fluor-JAK2 813 peptide, 10 nM rhodamine 110, and 0.5 μM GST−SH2-Bβ (525−670) (trace 1) or cell lysate from *E. coli* overexpressing the protein (trace 2). IS indicates rhodamine used as internal standard. Protein concentrations were similar in both samples. Separation conditions were the same as in Figure 5.

6 illustrates the division of a typical electropherogram into regions A1, A2, and A3, corresponding to free peptide, complex, and peptide dissociated from complex, respectively, using the criteria established for NECEEM.[5] Using these peak areas and eqs 2 and 3, K_d and k_{off} were determined to be 101 ± 12 nM and 0.95 ± 0.02 s^{-1}, respectively, based on three experiments with different concentrations of protein (0.5, 1, and 2 μM). The K_d is in reasonable agreement with the result from FA experiments (K_d = 82 nM) (Figure 2). The slightly higher value obtained by CE could be due to destabilization of the complex by heating within the capillary during separation;[27] although measurement of current as a function of voltage was linear in this range, thus providing no evidence of heating under these conditions (data not shown). The k_{off} corresponds to $t_{1/2}$ of 0.73 s for the complex. The dissociation rate of this binding system has not been determined previously, but our result is consistent with the general results for SH2 domain protein and phosphopeptide binding with k_{off} being 0.1−1 s^{-1}.[15,18]

Besides purified protein, lysate from cells overexpressing GST−SH2-Bβ (525−670) was also investigated by APCE-LIF. Figure 7 shows APCE results for samples containing 100 nM Fluor-JAK2 813 peptide and 0.5 μM purified GST−SH2-Bβ (525−670) (trace 1) or a 10-fold dilution of overexpressed cell lysate (estimated to be 0.5 μM based on FACE experiments). Interestingly, significantly more complex and less dissociated peptide was detected in the cell lysate sample. Using the same calculations, we found that the K_ds for lysate sample (104 nM) and purified sample (101 nM) were almost the same, whereas the half-life for the lysate sample ($t_{1/2}$ = 1.72) was 2.4 times that of the purified sample ($t_{1/2}$ = 0.73). This result suggests that protein within cell lysate has slowed on−off kinetics relative to purified protein. This may indicate the presence of other factors in cell lysate that alter kinetics of binding or it may be related to protein degradation effects after purification. Whatever the reason for the discrepancy, these results illustrate the potential of NECEEM for analysis of binding within complex mixtures.

(27) Buchanan, D. D.; Jameson, E. E.; Periette, J.; Malik, A.; Kennedy, R. T. *Electrophoresis* **2003**, *24*, 1375−1382.

APCE-LIF and FACE Assays for GST−SH2-Bβ (525−670) with BODIPY-JAK2 813 Peptide. We also evaluated the use of FACE and APCE for analyzing the interaction of GST−SH2-Bβ (525−670) with the BODIPY-JAK2 813 peptide. Because anisotropy measurements (Figure 2) indicated that BODIPY-JAK2 813 peptide has 5-fold lower affinity for GST−SH2-Bβ (525−670) than the fluorescein-labeled peptide, we anticipated a larger k_{off} and therefore more difficulty in detecting the complex by APCE. Figure 8 compares results of APCE and FACE experiments performed with BODIPY-JAK2 813 peptide similar to those performed with Fluor-JAK2 813 peptide as the fluorescent probe. The complex was not detected by APCE, although some broadening of the peptide peak was observed, likely due to interaction with protein (Figure 8A). We estimate that, with a separation time of 4 s and K_d of 100 nM, the highest k_{off} that can be determined by APCE is 2 s^{-1} corresponding to a $t_{1/2}$ of 0.35 s. In the FACE assay, the protein was readily detected (Figure 8B) with an LOD of 1 μM when using BODIPY-JAK2 813 peptide at 20 nM. The higher LOD, compared with Fluor-JAK2 813 peptide, is due to the lower fluorescence intensity of the BODIPY label, lower FA change upon binding, and lower binding affinity. From these results, we can conclude that APCE fails to detect complex with extremely fast off-rate; whereas FACE, in which separation is performed under equilibrium, is able to detect rapidly dissociating complexes.

Comparison of FA, FACE, and APCE. In these experiments, a protein−peptide binding interaction with fast kinetics has been investigated by FA, FACE. and APCE-LIF. Table 1 compares these techniques, in terms of their multianalyte capability, binding parameter quantification (K_d and k_{off}), molecular-size information, LOD, and sample consumption. K_ds were easily derived from anisotropy changes as a function of concentration. In principle, k_{off} could be determined by spiking an excess of unlabeled ligand and recording the decay of the complex by anisotropy; however, high dissociation rates require rapid mixing and recording capability. FA on a plate reader is unable to discern the presence

Table 1. Comparison of Analytical Capabilities for Detection of Noncovalent Interactions of Techniques Utilized in This Work

measurement property	FA	FACE	APCE
multiple analytes in one assay	no	yes	yes
K_d determination	yes	potentially	yes
k_{off} determination	potentially	potentially	yes
detect complex with rapid dissociation rate	yes	yes	limited by separation time
analyze crude samples (unpurified)	no	yes	yes
size information of complexes	yes	yes	no
sample consumed per assay at detection limit[a]	2.4 pmol	7.5 fmol	12 amol
LOD[a]	30 nM	300 nM	100 nM

[a] Sample consumption and LOD were determined from binding of GST−SH2-Bβ (525−670) and Fluor-JAK2 813 peptide. They can be different for other binding systems.

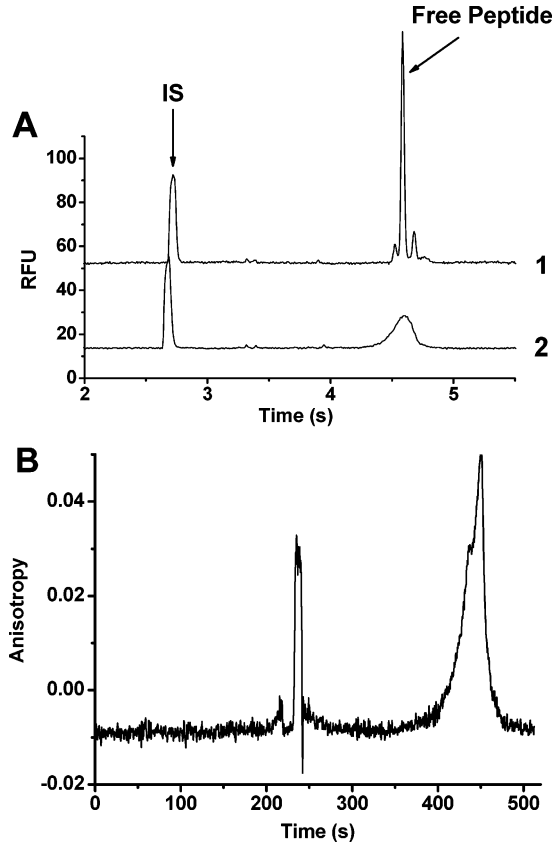

Figure 8. APCE-LIF and FACE assays of GST−SH2-Bβ (525−670) using BODIPY-JAK2 813 peptide as the probe. (A) APCE-LIF assays. Sample contained 50 nM BODIPY-JAK2 813 peptide, 6 nM rhodamine 110 (marked as IS), and 0 (trace 1) or 1 μM (trace 2) GST−SH2-Bβ (525−670). Separation conditions were as described in Experimental Section under APCE-LIF Assays. (B) FACE assays. Sample contained 5 μM GST−SH2-Bβ (525−670) and 20 nM BODIPY-JAK2 813 peptide. Electrophoresis buffer contained 20 nM BODIPY-JAK2 813 peptide. Separation conditions were the same as in Figure 3.

of multiple forms of the protein or peptide, and it is unsuitable for assays of cell lysate because of potential interference from viscosity and background fluorescence. The FA measurement provided the lowest concentration LOD but a relatively high mass LOD because a large amount of sample was consumed for a single measurement. An advantage of FA on a plate reader is its capability for high-throughput assays.

The main advantages of FACE in these experiments were its ability to partially resolve protein forms that interacted with the peptide and its ability to detect binding partners regardless of the off-rate (at least within the range used in this work) as illustrated by the experiment with the BODIPY-JAK2 813 peptide. FACE is also advantageous in that fluorescence anisotropy detection provides information on the size of the analyte, thus confirming the formation of complexes, and reducing the effect of background fluorescence on analyte detection in real biological samples when compared to APCE analysis. In principle K_d and k_{off} could be determined from FACE data since the anisotropy signal and peak shape depends on these parameters;[26] however, methods to extract this information have not yet been developed. The detection limit was worse than that obtained by FA; however, this is not an inherent limitation and may reflect instrumental differences in the fluorescence detection systems.

APCE was unique among the methods in demonstrating that multiple forms of the peptide ligand were present in the sample. In principle APCE could also separate multiple protein forms; however, this requires identification of conditions suitable for binding and separation, which is not always feasible. APCE is capable of measuring K_d and k_{off} when using the NECEEM method of analysis. Previous experiments measured off-rates on the order of $10^{-3}-10^{-2}$ s^{-1}.[5,6] In this case, we extended the capability to >1 s^{-1}; however the dissociation rate of the BODIPY-JAK2 813 peptide was apparently too fast to be detected. Thus, the time scale accessible by APCE is dependent upon separation speed and may be further improved with even faster separations. The LOD for GST−SH2-Bβ (525−670) was comparable to that of FACE, 100 nM corresponding to 12 amol injected. This detection limit is higher than previous reports for APCE[24] and may be due to the fluorophores, differences in detector design, and loss of complex during separation.

CONCLUSION

In this work, we investigated a binding system with rapid kinetics using two sensitive CE-based techniques, FACE and APCE-LIF. Using these two methods, binding interactions can be quantitatively studied, including interactions with rapid kinetics and within complex biological samples such as cell lysates. FACE and APCE-LIF may be useful complements to existing methods for studying signal transduction events and for discovering novel affinity interactions.

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