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Affinity capillary electrophoresis applied to the studies of interactions of a member of heat shock protein family with an immunosuppressant

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Abstract

The bioaffinity of receptor–ligand interactions is investigated by determining the binding constant (association constant or dissociation constant) of the resulting complex utilizing affinity capillary electrophoresis (ACE). The ACE binding assay was established with a potent immunosuppressant, deoxyspergualin (DSG), that binds specifically to Hsc70, a constitutive or cognate member of heat shock protein 70 (Hsp70) family. Quantitative determination of binding constants under different running buffer systems provide comparative results. The association constants for the interaction between Hsc70 protein and DSG were found to be $5.7 \cdot 10^4\ M^{-1}$ in a buffer with pH 6.95 and $6.3 \cdot 10^4\ M^{-1}$ in a buffer with pH 5.30 (or corresponding dissociation constants, 18 and 16 μM, respectively) based on Scatchard analyses. Binding of DSG to a synthetic peptide, SINPDEAVAYGAAV-QAAILSGDK, one of the DSG-binding fragments found from tryptic digest of Hsc70 protein, provides further detailed information for the understanding of Hsc70 binding domain. The applicability of using coated capillaries was also evaluated for probing Hsc70-DSG interaction.

1. Introduction

Heat shock (stress) proteins of the Hsp70 class and their cognates are a family of highly conserved cellular proteins. Their biochemical functions, although not well delineated, broadly include facilitating the binding to unfolded segments of a wide array of peptides and proteins, catalyzing folding and unfolding of certain proteins, and participating in their transport across intracellular membranes [1–4]. The induction of this protein family occurs when stressors cause cells to accumulate unfolded or abnormal proteins. The Hsp70 class consists of several members (M_r ranging from 70 000 to 78 000) that are induced at elevated temperatures or other forms of cell stress [5–7], and other members that are constitutively expressed include a M_r 70 000 heat shock cognate protein, termed Hsc70 [8,9]. In addition to the binding and ATP-dependent release of a wide variety of peptides and proteins and other biological functions, it has recently been found that constitutive Hsc70 protein in particular plays a role in immune responses [10–12]. Evidence has been found that a potent immunosuppressant, deoxyspergualin (DSG),

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whose mechanism of action remains unknown, binds specifically to Hsc70. This finding suggests that heat shock proteins may represent a new class of immunosuppressant-binding proteins, or immunophilins [12].

The understanding of specific recognition of a receptor by a ligand, such as protein–drug interactions mentioned above, is among the important aspects of biological studies in pharmaceutical research. The evaluation of bioaffinity of receptor–ligand interaction is usually performed by determining the binding constant (association constant or dissociation constant) between receptor and ligand through various biological assays and analytical means. Traditional biological binding assays, such as equilibrium dialysis, generally require radioactively labeled samples or relatively large amounts of sample, which is not always possible due to insufficient labeling and sensitivity. Other analytical approaches involve monitoring the change in UV absorption, fluorescence intensity, nuclear magnetic resonance, and the transition temperature in differential scanning calorimetry.

As an alternative approach, affinity capillary electrophoresis (ACE) has recently been introduced for the study of receptor–ligand interactions and the determination of the binding constants of formed complexes [13–19]. Although different approaches have been presented for binding studies, such as protein–drug ligand, protein–sugar, DNA–peptide, peptide–drug and antibody–antigen, etc., the basic principle lies in the measurement of an altered electrophoretic mobility (or migration shifting) of the complexed species as compared to free ligand. ACE, as one of the various operating modes of high-performance capillary electrophoresis (HPCE), inherits its many advantages, such as high resolving power, high sensitivity, short analysis time, quantitative abilities and instrumentation automation. In addition, no radiolabeling is required and simultaneous determination of binding constants for each individual component within a sample mixture is also possible. The capillary column contains a running buffer solution consisting of buffer components and one or more suitable binding ligands that are added at various concentrations. The mobility change of a proper receptor varies with a change of ligand concentration in buffer solution. Scatchard analysis can be performed by measuring the migration shift resulting from the change in charge status before and after the formation of the complex.

The structural basis for the binding property of Hsc70 and DSG interaction has not yet been defined. The general approach to access the potential relevance of the association of Hsc70 protein with the immunosuppressants and any other peptides will require the analysis of their affinity interactions and quantitative determination of the binding constants. We have previously utilized ACE to study the interactions of a small molecular ligand with peptidoglycan precursors [20]. In this paper, we examine the feasibility of using ACE for studying the binding behavior of a macromolecule, Hsc70 protein, a member of heat shock protein Hsp70 family, and its peptide fragment with a potent immunosuppressant. Here we demonstrate the utility of a rapid binding assay using the highly sensitive ACE approach for the quantitative determination of thermodynamic association constants (or dissociation constants) without radiolabeling, which may provide useful insights for understanding the function of heat shock proteins in immunological processes.

2. Experimental

2.1. Apparatus

ACE experiments were performed in both untreated fused-silica capillaries (Polymicro Technology, Phoenix, AZ, USA) and coated columns (supplied from J & W Scientific, Folsom, CA, USA) with 50 μm (or 75 μm) I.D. and 360 μm O.D. and effective separation length of 20–51 cm. The detection window was placed at about 7 cm from the end of the outlet. A Beckman P/ACE Model 2100 CE system (Beckman Instruments, Fullerton, CA, USA) equipped with both UV–Vis and laser-induced fluorescence (LIF) detectors was used. The capillary chamber was temperature controlled at 25°C by employing a liquid coolant. Pressure injection mode was employed throughout all

experiments. The components were monitored with the UV detector at a wavelength of 214 nm. Data were collected and analyzed with Beckman System Gold software.

2.2. Materials and reagents.

Deoxyspergualin (DSG) is a synthetic derivative of naturally occurring spergualin and was purified in the laboratory. Mesityl oxide was obtained from Aldrich (Milwaukee, WI, USA). 3-Hydroxytyramine was purchased from Sigma (St. Louis, MO, USA). All other reagents for buffer preparation are HPLC grade.

The constitutive heat shock protein Hsc70 was prepared from Jurkat cell (human T cell) and affinity purified as previously described [12]. Briefly, Jurkat cells ($1 \cdot 10^9$) were washed with phosphate-buffered saline and suspended in an affinity buffer. The cells were lysed by sonication and centrifuged. The supernatant was applied to an affinity column and washed with the affinity buffer at a flow-rate of 3.0 ml/min, followed by a linear gradient of 0.15 to 1 *M* NaCl in the buffer. Following the gradient, the bound protein was eluted with 5 m*M* DSG. The protein solution was further concentrated prior to electrophoretic experiments. The final concentration of protein for injection was not determined, which is not crucial for the measurement of binding shifts.

The DSG-binding peptides were identified from the tryptic digest of Hsc70 protein [12]. One of these peptides, SINPDEAVAYGAAV-QAAILSGDK, was synthesized using an Advanced ChemTech Peptide Synthesizer Model 200 (Louisville, KY, USA). The peptide was purified using a Waters μBondapak C_{18} analytical column (300 × 3.9 mm) with a mobile phase consisting of 0.1% trifluoroacetic acid in water–acetonitrile. A Beckman System Gold HPLC system was used for the purification and the peak was monitored at 214 nm. A stock solution with concentration of 1 mg/ml in water was prepared for subsequent ACE experiments.

2.3. Procedures

All binding assays were performed using an open-tubular HPCE system. Three operating buffers were prepared in this study. One buffer consists of 20 m*M* sodium borate/20 m*M* sodium phosphate at pH 6.95 and another buffer consists of 25 m*M* ammonium phosphate with pH 5.30. The third buffer consisting of 10 m*M* sodium phosphate (pH 2.80) was prepared for use in coated capillaries. A solution of Hsc70 protein was directly used for injection without further purification. The DSG-binding peptide solution was properly diluted for injection. The neutral marker, mesityl oxide dissolved in methanol (stock solution 5.0 mg/ml), was utilized to serve as a reference for the measurement of relative migration times in uncoated capillaries as reported previously [13]. An amine compound, 3-hydroxytyramine, was used to serve as a reference in the coated capillary column. DSG was dissolved in water at a concentration of 13.4 m*M* as stock solution, which was subsequently added into the running buffers at various concentrations. An aliquot of Hsc70 protein solution (or peptide solution) was mixed with an aliquot of reference marker and the running buffer solution for injection. The concentration of reference marker was properly adjusted to obtain a measurable reference peak. Pressure injection was utilized with a duration time of 3–10 s. Affinity interactions between Hsc70 protein (or peptide fragment) and DSG were examined through the measurement of relative migration shifting. The determination of binding constants was carried out by preparing a series of running buffers containing appropriate concentrations of DSG. Scatchard analysis illustrates the relationship between ligand concentrations and receptor migration times.

3. Results and discussion

Previous studies have identified the binding protein of DSG, a synthetic analogue of spergualin possessing potent immunosuppressive activity, as a member of the Hsp70 family [12]. Quantitative study of their relevance by determining the association constant has not yet been reported. We, therefore, chose this system as a model to evaluate the applicability of ACE for studying the receptor–ligand interactions, which

may provide information for the understanding of the biological function of Hsc70 protein in immunological processes. Initial experiments were performed using an untreated fused-silica capillary with an operating buffer consisting of 20 m*M* sodium borate/20 m*M* sodium phosphate carefully adjusted to pH 6.95. Previous studies have indicated that higher pH values may result in significant hydrolysis and degradation of DSG. It is known that protein adsorption to the capillary wall remains a general problem in the separation and analysis of proteins using HPCE techniques with untreated fused-silica capillaries and may be a major consideration in the measurements of migration shifting for biomacromolecules. General approaches to control this problem have included the use of coated columns whose inner surface was chemically modified, gel-filled capillaries, proper selection of running buffers, and various buffer additives. Two approaches have been employed in this study. The simple approach employs an uncoated capillary and a proper buffer to minimize the interference of column surface to the binding equilibrium between receptor and ligand. Adding various additives into the running buffer as commonly used in other CE modes is not recommended in this case since they may participate in the molecular interactions. Use of a coated capillary column may be an ideal solution to protein absorption and will be discussed later. The general indications of protein adsorption on the capillary surface in HPCE applications may include peak distortion and tailing, or even no peak elution at all. Under the present experimental conditions, It appears that protein absorption to capillary wall has been minimized to a great extent based on the elution of a sharp symmetric peak corresponding to Hsc70 protein as evidenced in Fig. 1. Therefore, we assume that protein adsorption may not be a problem in present experiments and has little effect on the measurement of affinity shift. In this study, the DSG was incorporated into the running buffer as one of the components. A neutral marker, mesityl oxide, was mixed with the protein and served as an internal reference. Monitoring of this DSG-binding protein at UV absorbance of

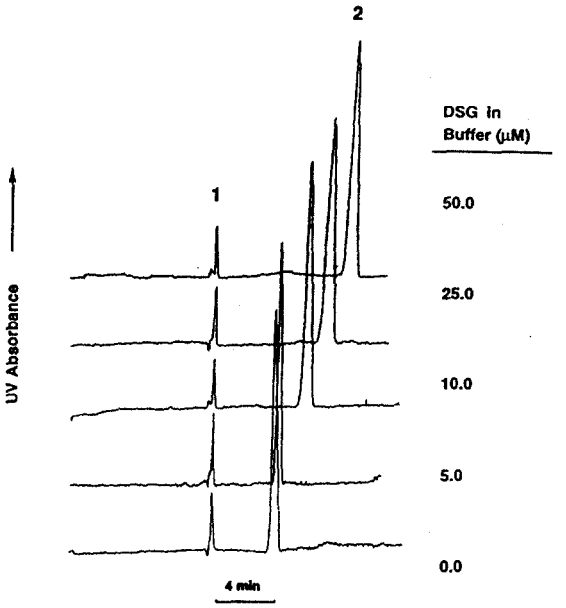

Fig. 1. Evaluation of DSG binding to constitutive Hsc70 protein by ACE. Peaks: 1 = neutral marker; 2 = Hsc70 protein. Buffer consists of 20 m*M* sodium borate/20 m*M* sodium phosphate (pH 6.95) containing DSG with concentrations ranging from 0 to 50 μM. Uncoated capillary: 56 cm (48.2 cm effective separation length) × 75 μm I.D.; sample: a mixture of Hsc70 and neutral marker pressure injected at 3 s; operating voltage: 20 kV (120 μA).

214 nm allows for the measurement of binding shifts using varying concentrations of DSG.

As displayed in Fig. 1, where the fixed concentrations of DSG range from 0 to 50 m*M*, the first peak corresponds to the neutral marker and the second peak corresponds to the DSG-binding protein, Hsc70, which gradually shifts away at higher concentrations of DSG from the neutral marker due to its interaction with the ligand. Since the DSG ligand is evenly distributed in the running buffer, introduction of the Hsc70 protein and subsequent migration behavior of this protein is determined by the electrophoretic mobility of its free form and the complex while interacting with the ligand at each point every moment inside the capillary column. Depending on the fixed concentrations of DSG, the charge status of the complex, and the binding kinetic rates of *on* and *off* processes, the change of the migration rates may result in measurable single-peak shifting and forms the basis for subsequent

Scatchard analysis. The data presented here clearly indicates that DSG binds to Hsc70 and results in a change of electrophoretic mobility, thus the shifting of migration times. The measurements of binding migration shift were repeated and the standard deviation under present conditions was found to be within 1.0%. The resulting Scatchard plot based on these data can provide an estimation of binding constant. With certain assumptions, such as the monovalent interaction between the receptor and ligand, achieved equilibrium during the electrophoretic run, and no significant alteration of the binding interaction associated with surface absorption of the capillary column, Scatchard equations were reported [14,15] for binding analysis in ACE experiments. These equations relate the ligand-induced incremental shift, $\delta\Delta t$, to the association constant K_b (or dissociation constant K_d, which is the reciprocal of K_b). A convenient form is given as following equation:

$$\frac{\delta\Delta t}{\delta\Delta t_s[\mathrm{L}]} = K_b - K_b \cdot \frac{\delta\Delta t}{\delta\Delta t_s}$$

or

$$R_f/[\mathrm{L}] = K_b - K_b R_f$$

where $\delta\Delta t_s$ is the relative migration shift of receptor at saturating concentration of ligand, [L] is the concentration of ligand, and $R_f = \delta\Delta t/\delta\Delta t_s$. A linear relationship between $R_f/[\mathrm{L}]$ and R_f is expected and the binding constant K_b is thus determined. As shown in Fig. 2, a linear Scatchard plot was obtained for the DSG–Hsc70 interaction. The association constant was determined under present conditions to be $K_b = 5.7 \cdot 10^4\ M^{-1}$ or dissociation constant $K_d = 18\ \mu M$.

In an attempt to examine appropriate conditions for the determination of binding constants, we studied several buffer systems and compared the binding results obtained in different operating conditions. A running buffer consisting of 25 mM ammonium phosphate adjusted to pH 5.3 was utilized. Under these conditions, Hsc70 protein behaves as a neutral species since the buffer pH is close to its isoelectric point value (pI = 5.2). There is no indication of protein absorption to the capillary wall, such as peak distortion and tailing. Thus, a series of running buffers containing DSG with concentrations ranging from 0 to 150 μM were prepared for the determination of binding constant and the results are displayed in Fig. 3. In the absence of DSG ligand, it was found that both Hsc70 protein and neutral maker coeluted, exhibiting a single peak since they both are neutral species thus are "dragged" out together by electroosmotic flow. As the DSG concentration is increased, a baseline separation of two peaks, or shifting of migration time, was observed as shown in Fig. 3. This phenomenon is most likely due to the binding interaction between DSG and Hsc70 protein. As a result of the change in migration rate, the protein was separated from the neutral marker which has no interaction with DSG. It seems that the interaction between the protein and ligand lacks significant change of net charge of the formed complex under these conditions, thus smaller migration changes are observed. A similar observation for an interaction of protein–sugar was also reported [15]. A linear Scatchard plot was obtained from 0 to 150 μM DSG for the Hsc70 protein as displayed in Fig. 4. Repeated experiments indicated that the standard deviation for the determination of relative

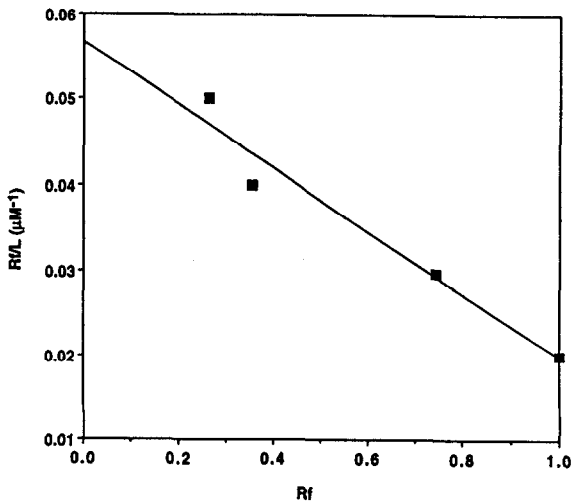

Fig. 2. Scatchard plot from data obtained in Fig. 1 for the determination of the DSG–Hsc70 binding constant at pH 6.95.

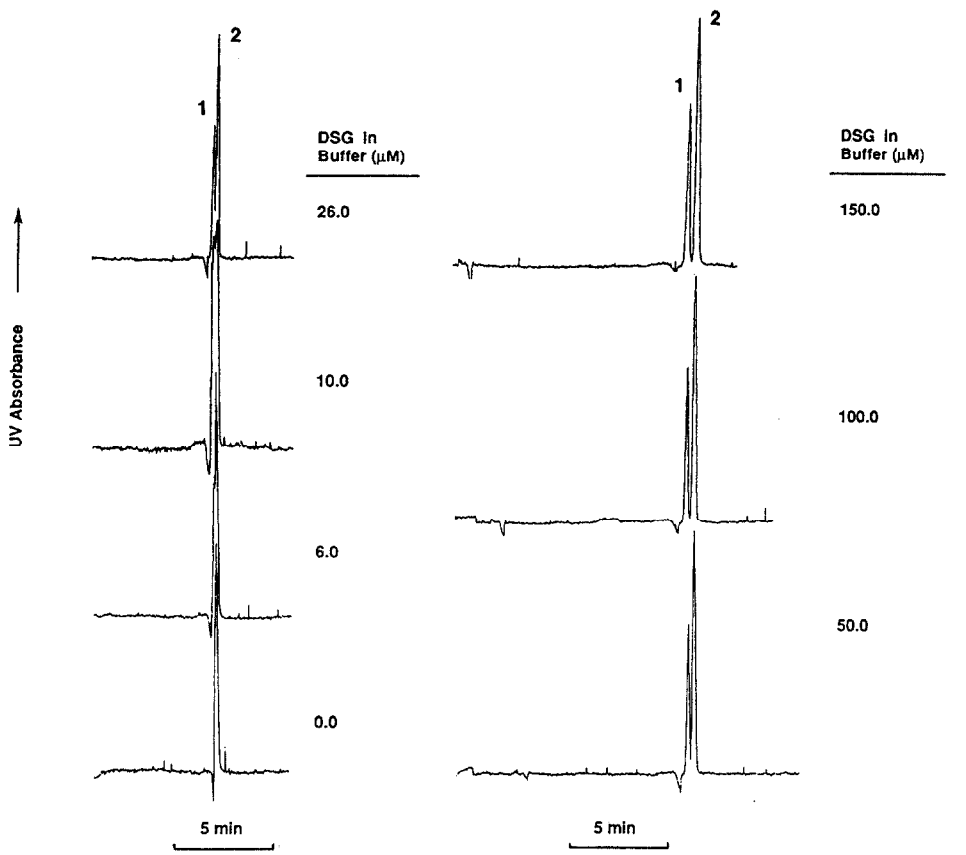

Fig. 3. Evaluation of DSG binding to constitutive Hsc70 protein by ACE. Peaks: 1 = neutral marker; 2 = Hsc70 protein. Buffer consists of 25 m*M* ammonium phosphate (pH 5.30) containing DSG with concentrations ranging from 0 to 150 μM. Uncoated capillary: 57.4 cm (50.7 cm effective separation length) × 50 μm I.D.; sample: a mixture of Hsc70 and neutral marker pressure injected at 10 s; operating voltage: 28 kV (28 μA).

affinity shifts is well within 1.0%. The value of association constant (K_b) was found to be $6.3 \cdot 10^4\ M^{-1}$ (or $K_d = 16\ \mu M$). These data compare favorably with those previously obtained under different running buffer conditions (where $K_d = 18\ \mu M$). These results also demonstrate the reliability of current methods for studying the binding behavior of receptor–ligand interaction and the determination of their binding constants.

Amino acid analysis of Hsc70 protein has revealed at least six DSG-binding fragments from its tryptic digest and their sequences have been determined to match well with those for the human constitutive Hsc70 [12]. To gain a better understanding of this interaction, a DSG-binding fragment (362–384), SINPDEAVAYGAAV-QAAILSGDK, was synthesized and studied by ACE to examine its binding behavior with DSG. Under similar experimental conditions to those described above, it was found that this peptide exhibits a strong shift of migration time as shown in Fig. 5. Further detailed studies on these DSG-binding peptides with the Hsc70 protein may provide useful information for the studies aimed at determining the structural relevance of their association and provide a better understanding of the DSG-binding domain.

Recent advances in the separation of bio-macromolecules, especially proteins, using HPCE techniques have been largely focused on controlling adsorption to the wall surface through various column approaches, such as gel matrix and surface coating. Chemical modifications of the silanol groups of the capillary wall

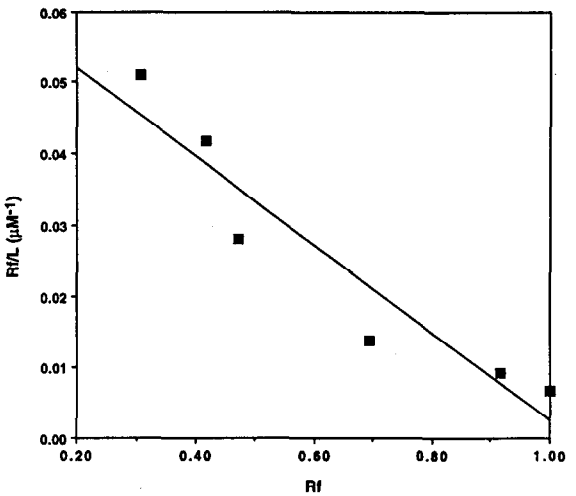

Fig. 4. Scatchard plot from data obtained in Fig. 3 for the determination of the DSG–Hsc70 binding constant at pH 5.30.

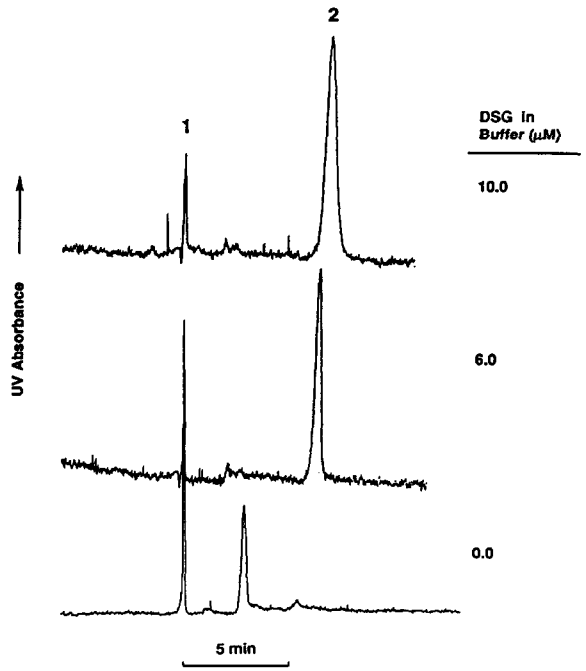

Fig. 5. Binding of DSG to a synthetic peptide corresponding to the fragment 362–384 of Hsc70 protein examined by ACE. Peaks: 1 = neutral marker; 2 = DSG-binding peptide (SINPDEAVAYGAAVQAAILSGDK). Buffer consists of 25 m*M* ammonium phosphate (pH 5.30) containing DSG with concentrations ranging from 0 to 10 μM. Uncoated capillary: 57.4 cm (50.7 cm effective separation length) × 50 μm I.D.; sample: a mixture of peptide and neutral marker pressure injected at 10 s; operating voltage: 28 kV (28 μA).

generally provide good reproducibility and peak shape for protein separations. Although protein adsorption has been minimized for Hsc70 at the selected operating pH values as discussed above, the capillary wall interaction with the protein has in no way been permanently eliminated. We have therefore investigated the usefulness of coated capillary columns for the binding studies. A coated fused-silica capillary of 27.6 cm (20.5 cm effective separation length) × 360 μm O.D. × 50 μm I.D. was used in this study. A series of phosphate buffers containing different fixed concentrations of DSG were prepared for the separation and affinity interaction. The neutral marker commonly used in uncoated capillary studies is no longer suitable in this case. The inherent electroosmotic flow, a migration driving force for the neutral marker in uncoated capillaries, was eliminated in coated columns. Therefore, a small molecule 3-hydroxytyramine, which was mixed with Hsc70 protein for injection, was selected as marker for the measurement of relative migration time. Generally, better reproducibility for protein separation was observed with a coated column versus uncoated capillaries. The standard deviation for the measurement of relative migration shifting is within 0.5%. Fig. 6 shows a series of binding behaviors between Hsc70 and DSG as a function of DSG concentration. The reference marker is expected to possess a net positive charge and migrates faster under current buffer conditions. The migration of Hsc70 gradually shifts from the marker whose migration time remains almost constant with an increasing concentration of DSG. It was observed that the peak shape of Hsc70 changes and becomes broad as the ligand concentration increases. This phenomenon is expected and is most likely caused by slow interaction kinetics between protein and ligand, which results in partial migration retardation of protein molecules during their interaction with the ligand molecules. This effect may become profound in the intermediate ligand concentration before saturation. It was also noticed that an unknown minor peak appeared between the major protein peak and the neutral marker. The

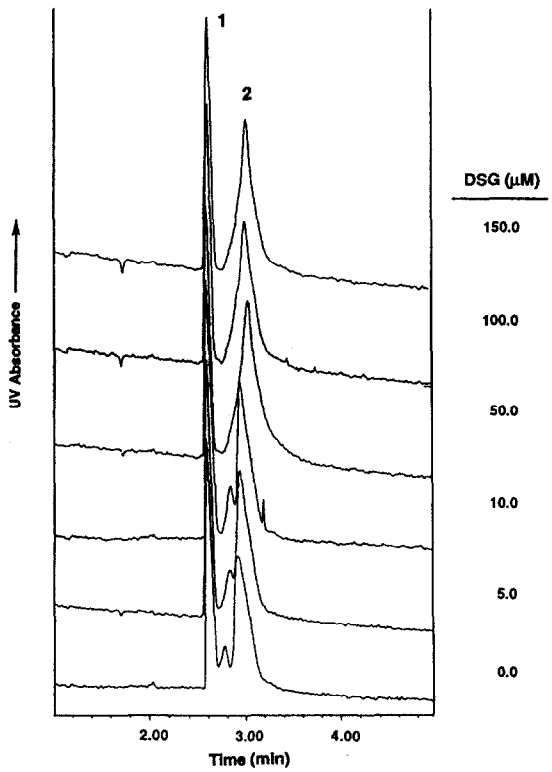

Fig. 6. Evaluation of DSG binding to constitutive Hsc70 protein by ACE in a coated capillary column. Peaks: 1 = marker; 2 = Hsc70 protein. Buffer consists of 10 m*M* sodium phosphate (pH 2.80) containing DSG with concentrations ranging from 0 to 150 μM. Coated capillary: 27.6 cm (20.5 cm effective separation length) × 50 μm I.D.; sample: a mixture of Hsc70 and reference marker pressure injected at 3 s; operating voltage: 15 kV (12 μA).

reason for this is not clear. The merging of these two peaks may be due to the change of protein peak shape (broadening in this case) as DSG concentration increases. This phenomenon seems to have little influence on the binding determination. These experiments represent examples which demonstrate the gradual changes of protein peak shape caused by the intermediate interactions between protein and ligand. This type of phenomenon has also been observed by other researchers [13,14]. Binding saturation occurs at a DSG concentration about 50 μm. The dissociation constant (K_d) was found to be 2.0 μM under present experimental conditions, somewhat lower than previously obtained data using uncoated capillaries. However, in those cases buffers with higher pH values were utilized to avoid protein adsorption. Currently, one of the major concerns of using coated capillary columns is perhaps the column coating stability which generally can not be recovered due to degradation. Certainly, more effort with regard to this aspect in column approach will need to be investigated in future studies.

The current development of ACE for the characteristics of ligand–biomacromolecule interaction represents an important direction of HPCE techniques whose applications have recently been expanding into many areas. The results presented here demonstrate the unique feasibility of ACE for the study of receptor–ligand binding due to speed, simplicity, flexibility and sensitivity. This may eventually result in new methodology with high promise for high-throughput screening of biomolecules and mechanism of action investigations in many areas of drug discovery research and pharmaceutical development.

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