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Investigations of cyclophilin interactions with oligopeptides containing proline by affinity capillary electrophoresis

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Abstract

Affinity capillary electrophoresis using mobility-shift analysis was utilized to characterize the binding of peptide ligands to cyclophilins, which are members of the enzyme family of peptidyl-prolyl *cis/trans* isomerases. Peptides derived from the human immunodeficiency virus capsid protein p24 exhibited different affinities to the isoenzymes cyclophilin18 and cyclophilin20. For the interaction of the peptide hormone bradykinin with cyclophilin18, a dissociation constant of 1.4 ± 0.1 m*M* was determined. Finally, the affinity of cyclophilin20 to peptides from a cellulose-bound peptide library scanning the sequence of *Drosophila melanogaster* protein cappuccino was investigated. The affinities of selected peptides to cyclophilin20 and a green fluorescent fusion protein with cyclophilin20 were compared.

Keywords: Affinity capillary electrophoresis; *Drosophila melanogaster*; Cyclophilins; Proteins; Peptides; Bradykinin; Cappuccino

1. Introduction

The recent progress in genomics and proteomics will further increase the knowledge of the function of proteins and may also guide the deeper understanding of the molecular cause of diseases. The binding of peptides as key ligands to a lot of proteins is therefore of great interest to researchers in biochemistry and pharmaceutical chemistry. The interaction of peptides with proteins may lead to very different actions, e.g. the digestion of the peptide or the modification of one of the binding partners leading to activation or deactivation within signaling pathways. Peptide sequences derived as putative binding sites or domains from proteins may also serve as markers to verify protein–protein interactions or to support the search for new drug leads.

Cyclophilins (Cyps) are members of the enzyme family of peptidyl-prolyl *cis/trans* isomerases (PPIases) that interact with a wide range of ligands including inhibitors [1], linear peptides [2–4], proteins [5,6] and DNA [7]. PPIases are also involved in the folding process of proteins [8–10]. The interaction of Cyps with the tight-binding inhibitor cyclosporin A (CsA) is known to play an important role in preventing graft rejection [11]. PPIases occur ubiquitously in cells [1,12] but their general function is not yet known in detail. The diversity of expected

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binding partners may suggest that the interactions are most often only transient with rather moderate to weak affinities.

Most of the methods utilized to verify protein–peptide interactions assume high binding affinities with a significant lifetime of the protein–peptide complex or require purified samples [13]. However, in the earlier stages of the search for interactions, moderate or weak affinities are actually more likely to occur. In these cases the applicability of methods, which need to immobilize or label binding partners may be limited.

According to its flexibility in separation conditions and robustness to buffer additives, affinity capillary electrophoresis (ACE) has been established as a powerful tool for the binding analysis of moderate and low affinity systems.

The aim of this work is to continue our efforts in the investigation of cyclophilin–ligand interactions using ACE. Recently, we reported about the Cyp interaction to its tight-binding inhibitor CsA and some CsA derivatives [14] and about the opportunity to use a green fluorescent protein (GFP) fused to Cyp to screen for Cyp–protein interactions [15]. In the present work we focus on interactions of Cyps to the peptide hormone bradykinin and to various peptides derived from protein sequences.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical grade. Water was triple distilled grade produced by a Milli-Q system (Millipore, Eschborn, Germany). Recombinant human cyclophilin18 (Cyp18) and recombinant cyclophilin20 from *Drosophila melanogaster* (Cyp20) were gifts from J. Fanghaenel and G. Kuellertz of our institute, respectively. Cyp20 fused to GFP (Cyp20–GFP) was prepared as described elsewhere [15]. Green fluorescent protein (GFP) was purchased from Clontech (Palo Alto, CA, USA). CsA was kindly provided by Novartis (Basle, Switzerland). Synthetic peptides derived from the human immunodeficiency virus (HIV) capsid protein p24 [16] Ac-DRVHPVHAGPZAPGQXREPRGSDIA-NH_2 (GP-peptide) and Ac-DRVHPVHA-GAZAPGQXREPRGSDIA-NH_2 (GA-peptide) as well as peptides selected by cellulose bound peptide libraries were provided by F. Bordusa from the synthesis group of our institute. Peptide sequences are given in single letter code. Additionally, Z symbolizes *p*-benzoylphenylalanine and X stands for norleucine. Ac- and -NH_2 stand for the acetylated and amidated N- and C-terminus, respectively.

The peptides Ac-AA and bradykinin were supplied by Bachem (Heidelberg, Germany). KCl, NaCl, DMSO, guanidinium hydrochloride, tris(hydroxymethyl)aminomethane (Tris), Tween 20, sucrose, NaH_2PO_4, Na_2HPO_4 and hydrochloric acid were obtained from Sigma (Deisenhofen, Germany).

Cellulose bound peptide libraries (peptide scans) were produced as described previously [17] and kindly provided by F. Bordusa from the synthesis group of our institute.

2.2. Apparatus

Capillary electrophoresis was performed on a 270A-HT system from Applied Biosystems (Foster City, CA, USA) and a P/ACE MDQ system (Beckman Coulter, Palo Alto, CA, USA). Data of the UV detector (270A-HT) were monitored and processed by a Kontron PC Integrator 3.90 (Kontron Instruments, Milano, Italy). P/ACE MDQ software 2.3 (Beckman Coulter) was used for data handling (P/ACE MDQ).

Fused-silica capillaries were provided by Polymicro Technologies (Phoenix, AZ, USA). UV detection was performed at 200 nm (270A-HT) or from 200 to 300 nm (P/ACE MDQ). Capillaries of 80 cm (length to the detection window 60 cm)×50 μm I.D. (270A-HT) or of 60 cm (length to the detection window 50 cm)×50 μm I.D. (P/ACE MDQ) were used. Operation voltage was 30 kV. Injection was performed hydrodynamically by a vacuum of 2.54 p.s.i. (16.9 kPa) for 3 s (270A-HT) or pressure of 0.5 p.s.i. (3.45 kPa) for 12 s for the samples and 0.5 s or 2 s for the internal standard, respectively. The capillary thermostating system was kept at 25 °C. Fluorescence imaging of the bound Cyp20–GFP on peptide scans was performed using an FLA-3000 scanner (Fuji, Tokyo, Japan) with an excitation and emission wavelength of 473 and 520 nm, respectively.

2.3. Methods

2.3.1. ACE

ACE was performed using appropriate peptide concentrations as buffer additives. A phosphate buffer (0.05 *M*, pH 8.0) served as background electrolyte (BGE). For the preparation of the ACE running buffers, stock solutions of the peptides (dissolved in BGE) were diluted to the final concentrations with BGE. Ac-AA (200 μ*M* in running buffer) and dimethylsulfoxide (DMSO, 3.5 μ*M* in running buffer) were used as internal standard and electroosmotic (EOF) marker, respectively, and were injected separately in addition to the sample. Samples of the investigated proteins were diluted from stock solution (buffer according to the final preparation step) with BGE to the final concentration of 10 μ*M*. In case of inhibition of the Cyps, the proteins were incubated with CsA solutions made by dilution in BGE from a stock solution in DMSO for 45 min at 4 °C. It was verified in preliminary experiments that the mobilities of the peptide ligands under investigation differ from the mobilities of the proteins. This is a precondition to successfully apply the mobility-shift approach.

2.3.2. Peptide scan analysis

A library of 13mer peptides linked covalently to a cellulose membrane was synthesized. This peptide library with overlapping sequences of 10 amino acids from spot to spot was used to move through the amino acid sequence of the *Drosophila melanogaster* protein cappuccino [18]. The following procedures were performed at 4 °C and with agitation of the cellulose membrane in the appropriate solutions. After synthesis, the cellulose membrane was rinsed with methanol and distilled water followed by an incubation in 8 *M* guanidium hydrochloride solution pH 7.5 for 30 min. This solution was diluted stepwise to 4, 2, 1, 0.5, and 0.25 *M* with binding buffer (0.03 *M* Tris pH 7.6, 0.17 *M* NaCl, 0.064 *M* KCl, 0.05% v/v Tween 20, 0.0015 *M* sucrose). After each dilution step the membrane was incubated for 15 min and finally rinsed three times with binding buffer for 30 min each. For binding, Cyp20–GFP was diluted from stock solution to 30 μ*M* using binding buffer and incubated with the membrane for 15 h. After three rinsing steps with binding buffer, the bound protein was directly detected on the membrane by fluorescence imaging using the GFP fluorescence of the fusion protein.

3. Results and discussion

Several ACE methods have been described for the determination of a wide range of dissociation constants [19,20]. For the investigation of intermediate and low affinity binding systems, the mobility-shift analysis is the method of choice because the analyte–ligand complex is only formed transiently. In order to obtain reliable data the sample concentration used for ACE experiments should match the range of the expected K_d value [21].

This means interactions with K_d values in the micromolar range fit to the sensitivity of common CE instruments using UV detection. The use of the ligand as a buffer additive causes increased UV absorption of the BGE leading to a decreased detection limit for the protein injected as the sample. However, this limitation is more severe for standard spectrometric assays. In mobility-shift analysis, the migration behavior of the analyte in the presence of the ligand is used to calculate dissociation constants [19]. Therefore, changes in the sensitivity do not influence the calculation of K_d as long as the peak of the analyte is still detectable. This offers the chance to determine K_d values of low affinity systems that are difficult to obtain with other techniques due to the required high concentration of ligand. Another advantage of ACE is the reduced consumption of ligand.

For the interaction of Cyps with peptides under investigation here, K_d values in the micromolar range are expected. Therefore, mobility-shift analysis with standard UV detection was employed.

3.1. Interactions of cyclophilins with peptide binding motifs of the HIV capsid protein p24

The binding of the HIV-1 capsid protein p24 to Cyps was found to be a crucial event in virus replication [22]. Recently, we demonstrated the applicability of ACE to this protein–protein interaction [15]. To introduce the ACE system for the binding of Cyps to peptide ligands, the known

interaction of Cyp18 to a 25mer peptide Ac-DRVHPVHAGPZAPGQXREPRGSDIA-NH_2 (GP-peptide) derived from the p24 sequence (amino acids 81 to 105) was used. The interaction of this peptide with exchanged amino acids in positions 91 and 96 with *p*-benzoylphenylalanine (Z) and norleucine (X), respectively, was already described in the literature. A K_d of 159±3 μ*M* obtained by a spectrometric test for the inhibition of the PPIase activity by the GP-peptide was published [23]. Therefore, this interaction was utilized as a reference system to establish the ACE method.

The GP-peptide was mixed with the buffer whereas Cyp18 was injected as the sample. The electropherograms in Fig. 1 demonstrate the influence of the peptide ligand on the migration behavior of rhCyp18 (lines A and C). Changes in migration time and peak shape of the Cyp18 peak are caused by interaction with the ligand during electrophoresis. The calculated electrophoretic mobility of Cyp18

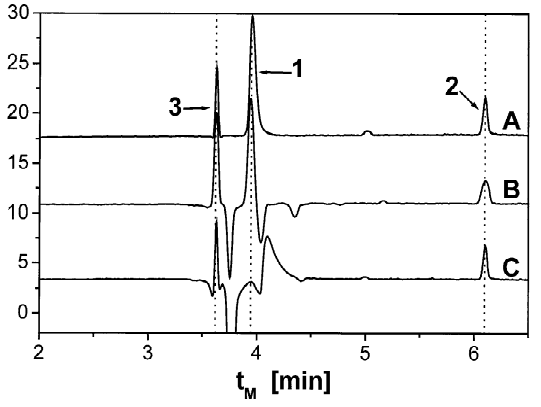


Fig. 1. Mobility-shift analysis of the interaction between Cyp18 and 25mer oligopeptides derived from HIV-1 capsid protein. Cyp18 (**1**) was injected in plain buffer (A) or in buffers containing 100 μ*M* of the peptides Ac-DRVHPVHAGAZAPGQXREPRGSDIA-NH_2 (B) or Ac-DRVHPVHAGPZAPGQXREPRGSDIA-NH_2 (C). Electropherograms were normalized (normalization was done for the better visualization of the effect caused by affinity and to filter the unspecific changes of the electrophoretic system due to different peptide concentrations present in the buffer. Hence, the migration times are normalized to the peptide-free buffer system. The electropherograms are set to have equal migration times between the markers DMSO and Ac-AA by means of factor multiplication for the observed migration time. This normalization did not influence any calculation of μ- or *K*-values) to the internal markers DMSO (**3**) and Ac-AA (**2**). Other conditions are given in the Experimental.

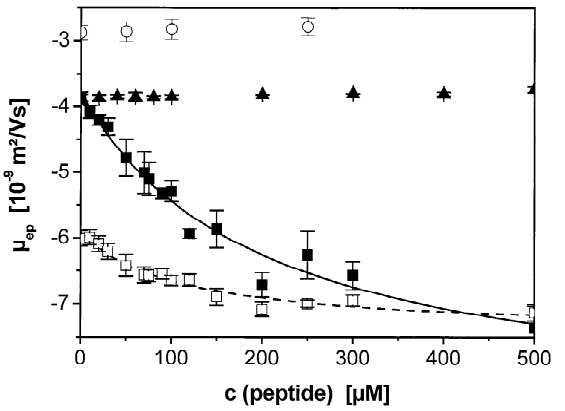


Fig. 2. Calculation of dissociation constants for the interaction of Cyp18 and Cyp20 to peptides derived from HIV capsid protein using mobility-shift analysis. The electrophoretic mobilities of Cyp18 (■) and Cyp20 (□) were plotted against the concentration of Ac-DRVHPVHAGPZAPGQXREPRGSDIA-NH_2 in the background electrolyte. Dissociation constants of 197±32 μ*M* and 106±18 μ*M* were determined using non-linear regression. The complex of Cyp18 with the tight binding inhibitor CsA [Cyp18–CsA] (○) served as control. Additionally, a variant of the peptide with an amino acid exchange in position 10 ($P^{10}A$) was used as a buffer additive (▲). All data points represent the mean of a triplicate measurement for μ_{ep}.

under the influence of GP-peptide was plotted against the concentration of the peptide in the BGE (Fig. 2 ■)

$$\mu = \mu^0 + (\mu^{max} - \mu^0) \cdot \frac{[L]}{K_d + [L]} \quad (1)$$

A dissociation constant of 197±32 μ*M* was determined from this plot by means of Eq. (1) [19], where μ represents the electrophoretic mobility of the protein at a certain concentration of the ligand *L*, μ^0 is the mobility of the unbound protein, μ^{max} symbolizes the mobility of the protein–ligand complex and K_d is the dissociation constant. The value for μ^0 is calculated from the initial experiments without ligand *L* in the BGE. The concentrations of the ligand *L* are known and the corresponding mobilities μ are measured. Using non-linear regression K_d and μ^{max} can be determined by fitting the experimental data of μ with Eq. (1).

The determined constant is in good agreement with binding data from the literature already mentioned [23].

Interaction studies for this system using Cyps and

peptides derived from the amino acid sequence of p24 clearly demonstrated the importance of the amino acid motif G^{89}–P^{90}. Exchange of one of these amino acids abolished the binding to cyclophilins for the protein and for derived peptides [22,23]. Therefore, it was tested if this changed binding behavior is also detectable by ACE. The addition of Ac-DRVHPVHAGAZAPGQXREPRGSDIA-NH_2 (GA-peptide) to the BGE did not cause changes in the mobility of Cyp18 in comparison to plain buffer (Fig. 1A and B). As expected, no changes of the electrophoretic mobility of Cyp18 with increasing concentrations of the GA-peptide in the running buffer up to 500 μ*M* were observed (Fig. 2 ▲). It is known that the binding of p24 and of derived peptides via the G^{89}–P^{90} motif is mediated by the active site residues of Cyp18 [24]. Hence, the tight-binding inhibitor CsA which blocks the active site of Cyps [1] is also able to inhibit the binding to the G^{89}–P^{90} motif of p24 [25]. Therefore, the complex [Cyp18–CsA] was formed by incubating of equimolar amounts of Cyp18 and CsA. Injecting this complex to the ACE system, no changes in its electrophoretic mobility in the presence of the GP-peptide were observed (Fig. 2 ○). This experiment also confirms that the observed mobility-shift for Cyp18 was caused by the interaction with the peptide in the BGE.

Furthermore, published data indicate that p24 binding occurs with several Cyp isoenzymes [26]. ACE was applied to investigate differences in the binding of the GP-peptide to Cyp isoenzymes. In the mobility-shift analysis of Cyp20, changes of the electrophoretic properties with increasing concentration of the GP-peptide in the BGE were detected too (Fig. 2 □). Obviously, the observed changes in the mobility of Cyp20 in the presence of the GP-peptide are smaller than for Cyp18. This indicates a reduced influence of the GP-peptide binding on the mobility of Cyp20 compared to Cyp18. A possible explanation for that observation may be the higher molecular mass of Cyp20 compared to Cyp18. This higher mass decreases the influence of the small peptide ligand on the mobility of the complex. Nevertheless, a K_d of 106±18 μ*M* was calculated demonstrating a tighter binding of Cyp20. Data from the literature for the full length p24 protein show the same trend for the K_d values of Cyp18 and Cyp20 using a spectrometric assay [27].

3.2. Interaction of Cyp18 with the peptide hormone bradykinin

Bradykinin is a peptide hormone of the sequence RPPGFSPFR that plays a role in blood pressure and temperature regulation [28,29]. The substrate binding of bradykinin to Cyp18 was described several years ago [30,31]. The affinity was postulated in the range of 10^{-3} *M* [30] but quantitative data are still missing probably due to the low affinity binding.

As shown in Fig. 3 (■), mobility-shift experiments clearly demonstrated changes in the mobility of Cyp18 in the presence of increasing concentrations of bradykinin. A K_d of 1.4±0.1 m*M* was calculated fitting the data of μ to Eq. (1) describing the binding quantitatively for the first time. The resulting K_d is in good agreement with the postulated value from the literature and fit also to values of other peptides binding as substrates to Cyp18 [2]. It is also known that the inhibitor CsA can block the interaction of Cyp18 and bradykinin [31]. Hence, the active site of Cyp18 is involved in the interaction with the peptide hormone [30]. The inhibition of Cyp18 with CsA that was investigated in ACE by

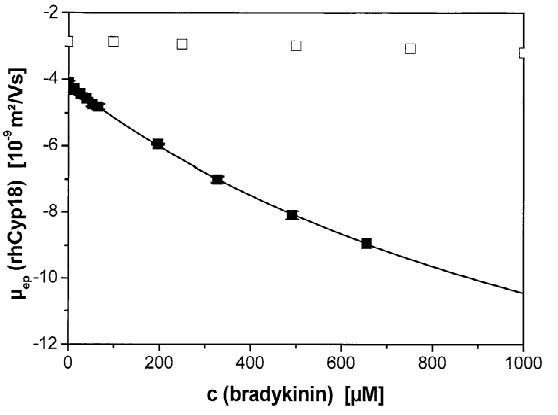


Fig. 3. Calculation of the dissociation constant of the Cyp18–bradykinin binding using non-linear regression. The mobilities of Cyp18 (■) and [Cyp18–CsA] (□) were plotted against the concentration of bradykinin present in the background electrolyte. For Cyp18 a dissociation constant of 1.4±0.1 m*M* was determined. The data points for Cyp18 represent the mean of triplicate measurements.

injection of the Cyp18–CsA complex instead of Cyp18 abolished the mobility-shift observed for free Cyp18 (Fig. 3 □).

Recently, we demonstrated the separation of Cyp18 and the Cyp18–CsA complex under similar CE conditions [14]. If Cyp18 is incubated with sub-equimolar concentrations of CsA, signals for free Cyp18 and the CsA complex are detected in one CE run. This is again shown in Fig. 4A. The equilibrium-mixture set-up was combined with the mobility-shift analysis to investigate the bradykinin interaction with Cyp18 and the Cyp18–CsA complex simultaneously. As illustrated in Fig. 4B the signal of the Cyp18–CsA complex remains unaffected while the peak of Cyp18 is shifted in its migration time. This indicates that an interaction of the bradykinin present in the buffer occurs with Cyp18 but not with Cyp18–CsA. This combined set-up leads to a reduced consumption of analysis time and material because interaction studies and controls can be performed simultaneously. Though this approach has wide application areas, the use is limited to systems that allow to distinguish between the species used for interaction and control, as demonstrated for free and inhibited Cyp18 in our case.

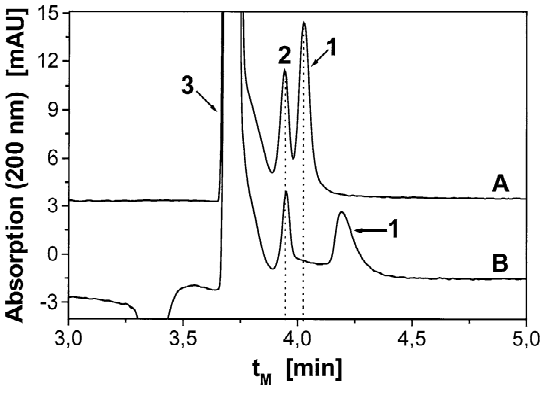


Fig. 4. Electropherograms representing the simultaneous investigation of the interaction of Cyp18 and bradykinin as well as the binding inhibition by CsA. By preincubating Cyp18 with sub-equimolar amounts of CsA both unbound Cyp18 (**1**) and Cyp18–CsA-complex (**2**) were present in solution. In plain background electrolyte (A) a separation of Cyp18 and [Cyp18–CsA] was achieved which is superimposed with the ACE interaction utilizing a buffer containing bradykinin (B, 100 μM). Electropherograms were normalized (see Fig. 1) to the internal markers DMSO (**3**) and Ac-AA (not shown). Other conditions are given in the Experimental.

3.3. Interaction of Cyp20 with peptides derived from cappuccino

The above described experiments employed ACE to gain quantitative binding data for already known systems of interaction. But ACE is also applicable to detect new and unknown ligands of receptor molecules by the screening of libraries containing potential binding candidates [32]. Furthermore, it was demonstrated that this approach enables the screening as well as the simultaneous determination of dissociation constants with a set of ligands [33]. Although this application of ACE is highly straightforward it is not yet widely used because of its demand for sophisticated devices, e.g. the hyphenation to mass spectrometry. We therefore decided to decouple the selection process and the K_d determination utilizing a peptide scan analysis and subsequent ACE experiments. Peptide scans are widespread for fast epitope mapping [34] and substrate specificity testing [17] and are also helpful to check for binding sites on proteins [35,36]. A peptide scan consists of a set of peptides whose chronological order of amino acids move stepwise through the amino acid sequence of the complete protein. Although the results of peptide scan binding tests can only give a suggestion of putative binding sites in a protein, because they do not consider the three-dimensional protein structure, it is a fast, cheap, and easy way to detect possible interactions. The major advantage is that the full-length protein is not necessary, which is especially important in cases where proteins are difficult to obtain due to problems in expression or purification.

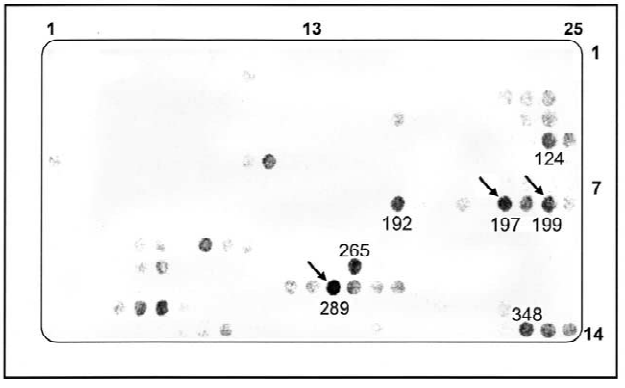
Recently, the interaction of Cyp20 with a putative ligand in *Drosophila melanogaster* lysate was demonstrated by ACE [15]. This work was now pursued to gain hints about the possible binding partner. The protein cappuccino was suspected as a potential ligand because of the high content of proline residues in the amino acid sequence of the protein [37]. The recognition of proline-containing binding motifs is a fundamental property of the PPIases [1]. Cappuccino is a protein from *Drosophila melanogaster* which plays an important role in early development stages

of the fruit fly [38]. The sequence of the protein was subjected to a 13,3 peptide scan, which means that 13mer peptides were synthesized and the amino acid sequence of the peptides moved three amino acids from spot to spot through the protein sequence from N- to C-terminus. To visualize the binding of the covalently membrane-linked peptides with Cyp20, a solution of a fusion protein with GFP (Cyp20–GFP) was directly incubated with the membrane. Peptide spots which interact with Cyp20 were easily detected by the fluorescence properties of the fusion part GFP using fluorescence imaging (see Experimental for details). This approach may provide an alternative to commonly applied detection methods which utilize specific antibodies but require an additional blotting step of the bound protein to nitrocellulose [17].

Fig. 5 illustrates the fluorescence image of the membrane after incubation with the fusion protein. The experiments were repeated on the stripped membrane as well as on another independently synthesized membrane to confirm the results (data not shown). The sequences of seven possible peptide binding motifs are listed in the table. They were selected on the one hand due to their fluorescence intensity on the spots. On the other hand one has to consider that positive binding spots should be accompanied by more or less fluorescing spots in the direct neighborhood. Binding motifs usually consist of 4–10 amino acids but the peptide sequence on the membrane only changes by three amino acids from spot to spot, whereas 10 amino acids are unchanged. Even an intense fluorescing single spot is therefore more likely to represent an artifact, if it is not escorted by other marked spots. The reliability of the results derived from the peptide scan analysis is further hampered for some other reasons. The real yield of the peptide synthesis may vary from spot to spot dependent on the particular peptide sequence. Furthermore, artifacts due to unspecific protein binding to the membrane surface could occur. In our special case, an additional problem may emerge from the use of GFP as detection label because peptides which bind to the GFP part of the fusion protein were detected as well.

Therefore, ACE as an independent analysis method was used to verify and quantify the binding to probable motifs selected from the peptide scan analysis. Three out of the seven candidates from the table in Fig. 5 were selected to limit synthesis expense (peptides **c**, **d**, **f**). The peptides **c** and **d** were chosen instead of other spots with similar intensity due to their sequence containing proline. Although peptide **f** has no proline in the sequence it could not be ignored because this spot shows the maximum fluorescence intensity (Fig. 5).

The ACE analysis for peptide **f** was not possible due to the poor solubility in aqueous solution. Firstly, the peptides **c** and **d** were tested for their binding to Cyp20–GFP. Although both peptides were binding motifs for Cyp20–GFP in the peptide scan



peptide	spot No.	sequence
a	124	NQKTLQQILKKRL
b	192	GNWFHRTNTMRKS
c	197	NPPKPMRPLYWTR
d	199	RPLYWTRIVTSAP
e	265	VTLLFRKLEVTSQ
f	289	HFIVRTYIAQRRK
g	348	SGRISLKERMLMR

Fig. 5. Identification of Cyp20–GFP binding to cellulose-bound peptides of a 13,3 peptide scan derived from the *Drosophila melanogaster* protein cappuccino. The bound fusion protein on peptide spots was detected by GFP fluorescence. Peptides **c**, **d**, and **f** were selected for further ACE experiments in solution. Conditions as stated in the Experimental.

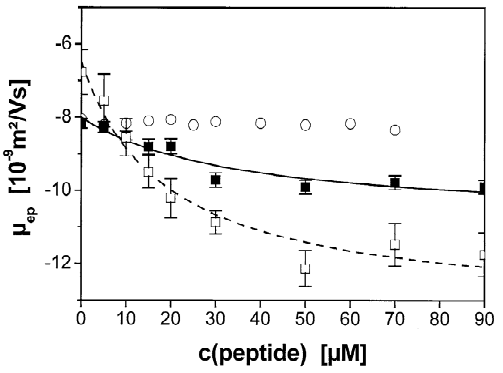


Fig. 6. Verification of the interaction of Cyp20 with peptides derived from the cappuccino sequence by ACE. Using mobility-shift analysis, the binding of Ac-NPPKPMRPLYWTR-NH_2 (■) and Ac-RPLYWTRIVTSAP-NH_2 (○) to Cyp20–GFP was investigated. For Ac-NPPKPMRPLYWTR-NH_2 a dissociation constant of 35±5 µ*M* was determined, whereas Ac-RPLYWTRIVTSAP-NH_2 caused no change of the mobility of the fusion protein. For Cyp20 (□) without GFP fusion the binding to Ac-NPPKPMRPLYWTR-NH_2 was quantified by a dissociation constant of 19±2 µ*M*. Other conditions are given in the Experimental.

only **c** gave rise to a mobility-shift in ACE (Fig. 6 ■) whereas **d** showed no effect (Fig. 6 ○). A possible explanation for the discrepancy of the results for **d** in peptide scan and ACE may be the N-terminal extension of the peptide with β-alanine during the linking to the membrane. This additional peptide bond which is not present in solution may stabilize the peptide to enable the binding to Cyp20–GFP.

For peptide **c** a dissociation constant of 35±5 µ*M* was determined (Fig. 6 ■). To exclude artificial binding effects caused by the fusion of Cyp20 to GFP the interaction of peptide **c** with Cyp20 without GFP fusion was investigated. Peptide **c** also caused a mobility-shift for the signal of Cyp20 (Fig. 6 □). A K_d value of 19±2 µ*M* was calculated from the data. The increasing binding affinity of peptide **c** to Cyp20 compared to Cyp20–GFP is probably due to the GFP fusion. The large protein label (about 27 kDa) may partially shield the binding site in Cyp20 thereby influencing the accessibility for the peptide. Finally, control experiments using GFP were done. As expected, no mobility-shift was observed for both peptides **c** and **d** (data not shown).

4. Conclusions

It was demonstrated that ACE in mobility-shift mode is an effective method to study the interaction of cyclophilins with peptide ligands. The utilization of UV detection provides the basis for the investigation of intermediate and even weak binding systems due to the universal applicability for peptides and proteins. In this affinity range, ACE can decrease the necessary consumption of material compared to other methods like isothermal titration calorimetry.

Proline motifs play an important role in binding to cyclophilins. The sensitivity of ACE to detect affinity changes due to the exchange of a single amino acid in the ligand was demonstrated for the binding of peptides derived from the HIV capsid protein to cyclophilins. This approach seems promising to study the influence of slight changes in peptide binding motifs regarding their interaction with proteins.

In the case of the low affinity binding of Cyp18 to the peptide hormone bradykinin, quantitative binding data were achieved. ACE was thereby proven to be an alternative method for the interaction analysis in this range of dissociation constants in vitro. Furthermore, the combination of both equilibrium-mixture and mobility-shift analysis in ACE allowed the simultaneous measurement of K_d values and control experiments as demonstrated for free and inhibited Cyp18. This can significantly decrease the analysis time and combines the gathering of binding data with information on the active site binding of the ligand.

Finally, the combination of ACE and peptide scan analysis provides an approach for the investigation of interaction with unknown ligands. Based on the library of cellulose bound peptides, binding studies for complete protein sequences or exchanges in peptides are possible. The subsequent analysis of identified peptides that bind to a receptor protein confirms and quantifies the suggested binding in solution.

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