A transient kinetic study between signaling proteins: the case of MEK-ERK interaction

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1. Introduction

In the last two decades biological research has been focused on the exploration of the basis of cell regulation and function at molecular level. We now know that cell responses to external stimuli are determined by intracellular intricate signaling networks, which are composed of biochemical pathways based essentially on protein interactions. Despite considerable progress has been made, it is apparent that the flow of biological information has not yet been fully deciphered. In particular the abundance of qualitative information, concerning cellular interacting components, needs to be improved with sufficient availability of quantitative data. In this context, the set-up of approaches to provide high quality data in terms of the determination of kinetic parameters is needed to contribute to this kind of improvement. This is of special interest to systems biologists who deal with the development of kinetic model of intracellular signal transduction and often suffer from the lack of available kinetic parameters.

Central building blocks in the intracellular signalling networks are the mitogen-activated protein kinase (MAPK) cascades which are critical for cellular decision to proliferate, differentiate, or undergo apoptosis.1-3 MAPK cascades comprise several set of cytoplasmic protein kinases organized as modular pathways. The activation of upstream kinases by cell surface receptors leads to sequential activation of an evolutionarily conserved core module of protein kinases (MAPKKK → MAPKK → MAPK).4 The most widely characterized mammalian MAPK cascade is the Raf–MEK–ERK axis (Fig. 1) along which multiple isoforms are present at each level. Among them, two high related isoforms, MEK1/2 and ERK1/2 play a primary role in signal transduction.

Although the MAPK cascades have been the subject of intense research, there are few quantitative kinetic studies on the components belonging to this pathway. In particular the association and dissociation rate constants (i.e., $k_{on}$ and $k_{off}$, respectively) for the binary protein–protein interactions in this cascade have been derived from simulation/prediction studies5-6 or from in vitro studies of the kinase enzymatic reactions.7-9 There is only one recent work10 in which the dissociation binding constants ($K_d$) concerning MAPK complexes have been estimated from measurements on living cells. Therefore in vitro accurate determination of the kinetic parameters seems to be necessary to provide a more complete knowledge, allowing a direct comparison with the data obtained by different methodologies at the same time.

Numerous techniques have been described and applied to characterize protein–protein interactions in vitro,11-13 These techniques are often specialized and frequently use proper fluorescent probes as versatile means for studying the kinetics of protein interactions. Stopped-flow fluorescence intensity method offers a powerful tool for detailed kinetic analysis of protein interactions and has gained considerable importance during the past few years.14 The main advantages of this technique are the following: (i) the reaction can be monitored continuously in real time over a broad range of reactant concentrations at the millisecond time scale; (ii) the system operates in a liquid environment where the interacting molecules are not bound to a solid support, this provides a more physiological condition of work, minimizing possible measurement alterations due to conformational changes that could arise from the protein-support binding; (iii) the experiments are not time-consuming.

The present study reports the kinetic investigation of the interaction between MEK and ERK by stopped-flow fluorescence...
Glutathione S-transferase (GST)-tagged protein, expressed in MAPKK, MEK (two isoforms, MEK1/2), by phosphorylation. Following the activation of the MAPKK, Raf-1. Active Raf-1 binds and activates the growth factor receptor (GFR) activation, which transmits the signal by the Raf/MEK/ERK axis. The signaling via this cascade is usually initiated by values for the fluorescence of its homemade recombinant MEK and ERK proteins. Here kinetic derivation from the analysis of the kinase enzymatic reaction. The commercial recombinant MEK and ERK proteins used were 2.1 Reagents

2. Experimental Procedures

2.1 Reagents

The commercial recombinant MEK and ERK proteins used were full-length active MEK1 and full-length inactive ERK1. MEK1 was purchased from Upstate® as N-terminal GST and C-terminal 6His-tagged, expressed in E. coli, purified using glutathione-agarose; purity 97%; MW = 70 kDa. The homemade recombinant MEK and ERK proteins used were the constitutively active mutant MEK1 R4F (containing an N-terminal deletion of aa 32-51 and the Ser218Glu/Ser222Asp substitutions) and full-length inactive ERK2; both the proteins were produced in the laboratory of the authors as follows. MEK1 R4F and ERK2 were expressed in the BL21-DE3 strain of E. coli transformed with pGEX-KG MEK1 R4F and NpT7-5 ERK2, respectively. MEK1 R4F (expressed as GST-fusion protein; MW = 66 kDa) and ERK2 (expressed with 6His-tag; MW = 42 kDa) were purified as described in detail previously,55 minimum 90% purity as determined by Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie blue (Bio-Safe Coomassie, Bio-Rad) staining. Purified MAP Kinases were dialyzed against Dulbecco’s Phosphate-Buffered Saline (PBS; BE17-512F; BioWhittaker/Cambrex) using Slide-A-Lyzer 3.5K (PIERCE).

MEK1 R4F and ERK2 concentrations were determined after resolving recombinant protein samples and dilution series of purified 2mg/ml bovine serum albumin by SDS-PAGE and staining with Coomassie blue; the gels were then documented using ChemiDoc XRS charged-coupled device (Bio-Rad) and the proteins were quantified using Quantitative One software (Bio-Rad).

ERK2 and MEK1 R4F expression plasmids were a generous gift of Melanie Cobb (University of Texas, Southwest) and Natalie Ahnn (University of Colorado, Boulder), respectively.

Fluorescein-5-maleimide was synthesized as reported previously.16

2.2 Bioconjugation

ERK proteins have been bioconjugated with fluorescein-5-maleimide following the reported protocol.17,18 ERK concentration was 0.4 mg/ml and the excess of fluorescein-5-maleimide used was 50-fold. After bioconjugation, the derivative was immediately purified using Sephadex® G-25 desalting column (Amersham Bioscience) and PBS (pH=7.4) as eluent.

2.3 Calculation of Dye/Protein ratio

To evaluate the fluorophore labeling efficiency, the dye/protein ratios (D/P) of the conjugates were determined by the absorption spectra of the labeled proteins, registered in PBS (pH=7.4) according to the relationship reported in eqn (1):

\[
D / P = \frac{A_{\text{max}} \cdot \varepsilon_{\text{prot}}}{(A_{280} - cA_{\text{max}}) \cdot \varepsilon_{\text{dye}}} \quad (1)
\]

where \(A_{280}\) is the absorption of the conjugate at 280 nm; \(A_{\text{max}}\) is the absorption of the conjugate at the absorption maximum of the corresponding fluorescein-5-maleimide; \(c\) is a correction factor which must be used to adjust for amount of \(A_{280}\) contributed by the dye because fluorescent dyes also absorb at 280 nm and equals the \(A_{280}\) of the dye divided by the \(A_{\text{max}}\) of the dye (\(c = 0.29\)); \(\varepsilon_{\text{prot}}\) (42230 cm\(^{-1}\) M\(^{-1}\)) and \(\varepsilon_{\text{dye}}\) (63096 cm\(^{-1}\) M\(^{-1}\)) are the molar extinction coefficients for the protein used and fluorescein-5-maleimide, respectively. The evaluation of \(c\), \(\varepsilon_{\text{dye}}\) and \(\varepsilon_{\text{protein}}\) were calculated as reported elsewhere.19,20
ERK presents ten cysteine residues and the resulting $D/P$ value was 0.98 for both commercial and homemade proteins on the basis of three averaged bioconjugations.

2.4 Experimental techniques

UV-Vis measurements for the evaluation of the fluorophore labeling efficiency were recorded using a Shimadzu UV-1700 Pharma Spec Spectrophotometer equipped with 1.0 cm path length quartz cells.

Fluorescence kinetics measurements were recorded using an Applied Photophysics SX20 stopped-flow spectrometer fitted with a 515 nm cut-off filter between the cell and fluorescence detector and equipped with a thermostat bath (30°C). Data acquisition, visualisation and analysis were provided by Pro-Data software from Applied Photophysics Ltd.

2.5 Experimental procedures

Biolabelled ERK concentration (10 nM in PBS) was kept constant and several shots of different MEK concentrations were performed: 100 nM, 120 nM, 140 nM and 160 nM in PBS for the commercial proteins. For homemade production, the chosen MEK concentrations were: 20 nM, 40 nM, 60 nM, 100 nM and 130 nM. The reported concentrations are syringe concentrations, this means that the real concentrations in the cuvette are halved. Each experiment (whole concentration set) was repeated five times, each time using a new mother solution and a different set of protein production. For each dilution condition at least five scans were acquired and averaged. Each experimental point is therefore an average of 25 shots. Raw data were analyzed and plotted to a single exponential function by using Pro-Data Viewer 4.0.17 and from this data treatment the observed rate constants ($k_{obs}$) were obtained. The excitation wavelength was 495 nm and slits widths of the excitation monochromator were 0.2 mm.

The displacement experiment was performed according to Eccleston et al. A solution containing a mixture of 50 nM biolabelled ERK with 50 nM MEK was rapidly mixed with a large excess (400 nM) of unlabelled ERK. The displacement was repeated four times and the $k_{off}$ obtained is an averaged value of these four experiments.

3. Results

To study the interaction between MEK and ERK we firstly used the commercial proteins. The binary protein binding was investigated under pseudo-first order conditions (i.e., [MEK] >> [ERK]). Fig. 2 shows that on mixing 10 nM ERK with 120 nM MEK there is a decrease in fluorescence intensity which can be well fitted to give a rate constant of 12.49 s$^{-1}$.

The dependence of the $k_{obs}$ for MEK-ERK binding was investigated using 10 nM biolabelled ERK and the range of 100-160 nM MEK (Fig. 3). In these experimental settings, the values of the kinetic parameters can be calculated from the slopes and intercepts of the linear plots of $k_{obs}$ versus increasing concentration of MEK (see eqn 2).

$$k_{obs} = k_{on}[MEK] + k_{off}$$  \hspace{1cm} (2)

In particular, the slope of the straight line is the $k_{on}$ (second-order rate constant; units, M$^{-1}$ sec$^{-1}$) and the intercept on the ordinate is the $k_{off}$ (first-order rate constant; units, sec$^{-1}$). The second-order rate constant $k_{on}$ is 5.49·10$^8$ M$^{-1}$s$^{-1}$. As it can be seen, the intercept on the ordinate has a small negative value (-24 sec$^{-1}$). This shows that the interaction is nearly completely shifted toward the formation of the complex.
sec$^{-1}$). This shows that the interaction is nearly completely shifted toward the formation of the complex.

To further investigate this aspect, we then used more cheap homemade proteins in order to perform other interactions and to carefully evaluate the $k_{off}$ value. The interaction was repeated and the dependence of the $k_{off}$ for MEK-ERK binding was again investigated using 10 nM biolabelled ERK and a range of 20-130 nM MEK (Fig. 4). The slope of the straight line through the points gives the apparent $k_{on}$ value and this is estimated to be $3.23 \times 10^{8}$ M$^{-1}$s$^{-1}$ which is in good accordance with the previous value obtained with commercial proteins. The intercept of the straight line with the $y$-axis should give the $k_{off}$ value, but again it cannot be reliably distinguished from zero with the obtained data. It should be noted that there will be a significant error on the points at low concentrations, since true pseudo-first-order conditions did not prevail. The intercept would not be reliable even if seen to be finite but small.

Since disassociating rate constant value seems to be small, obtained $k_{off}$ is not reliable. To gain more information and to measure $k_{off}$ value accurately, a displacement experiment was executed as proposed previously. Briefly, in the displacement experiment (see Scheme 1), a solution containing fluorescent ERK (ERK-nL) and MEK is mixed with an excess of non-fluorescent ERK (ERK-nL). The concentration of ERK-nL is chosen to get a saturation of MEK, and then a high concentration of ERK-nL is added so that ERK-nL dissociates from MEK and cannot reassociate. In this way the rate constant of the observed process is determined only by $k_{off}$.

The $k_{off}$ value was therefore measured directly by displacement of ERK from its complex with MEK using an excess of unlabeled ERK. Fluorescence intensity was monitored during the process and the corresponding record was fitted to give an averaged rate constant of 18.7 s$^{-1}$ (Fig. 5). This $k_{off}$ value together with that obtained for the $k_{on}$ allowed us to calculate the $K_{d}$ value of 58 nM for MEK-ERK interaction. This is in excellent agreement with the $K_{d}$ value of 50 nM obtained by steady-state fluorescence in the same buffer (data not shown).

![Fig. 4 Dependence of the $k_{on}$ determined by fluorescence intensity of the binding of ERK to increasing MEK concentrations (homemade proteins). Data points are the mean of five independent experiments ± standard deviation. Error bars not visible are concealed by the data point.](image)

![Scheme 1 Displacement reaction.](image)

![Fig. 5 Stopped-flow fluorescence record of a representative displacement experiment (homemade proteins): one syringe contained 50 nM biolabelled ERK, 50 nM MEK, and the other syringe contained 400 nM unlabelled ERK. The plot shows a fit to a single exponential with a rate constant of 20.5 s$^{-1}$. The experiment was repeated four times and the averaged obtained value is 18.7 s$^{-1}$ ± 2.2.](image)

### 4. Discussion

In the present work, the binding between MEK and ERK, two members of a crucial intracellular signal pathway, was studied by stopped-flow fluorescence intensity. The values of $k_{on}$ and $k_{off}$ were experimentally measured for the first time avoiding their derivation from the analysis of the kinase enzymatic reaction.

$K_{d}$ value was determined from the $k_{off} / k_{on}$ ratio and results to be in excellent agreement with the values obtained in previous in vitro studies. This shows that the use of stopped-flow technique is a suitable approach for studying protein-protein interactions in vitro.

A careful collection of available information, relative to the kinetic parameter for MEK-ERK interaction, allowed us to compare the values obtained with those previously found by different methods (Table 1). In fact, the $K_{d}$ value reported is of the same order of magnitude of that obtained by deriving $k_{on}$ and $k_{off}$ from the in vitro study of the kinase enzymatic reaction. Compared to those obtained by in vitro methods, the $K_{d}$ values derived from simulation modeling by the group of Schoeberl and Yamada result to be one and two order of magnitude different, respectively. The in vitro $K_{d}$ value results to be one order of magnitude different from that obtained in vivo by Fujioka and coworkers. The authors calculated the half-life of the MEK-ERK complex, and estimated the dissociation rates from these half-lives.

### Table 1 Parameters for MEK-ERK interaction.

<table>
<thead>
<tr>
<th>references</th>
<th>method</th>
<th>$k_{on}$ (10$^9$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_{d}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>in vitro</td>
<td>323</td>
<td>18.7</td>
<td>58</td>
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Bhalla 2004a,
Sasagawa et al. 2005a
Fujisawa et al. 2006
Schoeberl et al. 2002
Yamada et al. 2004

"Considering the Michaelis-Menten formulation as a special case of two reactions in sequence (Bhalla & Iyengar 1999), the values corresponding to $K_a$, $k_{on}$ and $k_{off}/k_{on}$ ratio (available in the DOQS database, http://doqs.ncbs.res.in) for MEK-ERK kinase enzymatic reaction allowed the derivation of the values corresponding to $k_{on}$, $k_{off}$ and $K_d$ for MEK-ERK binding.

In the context of protein-protein interaction it is well established that the in vitro study requires in vivo validation, and vice versa. Measures performed in living cells have to be considered of high value due to the physiological/cellular context in which molecules operate. On the other hand, in vitro methods analyze binary protein-protein interactions in a cell free system, which is characterized by the absence of positive or negative influence exerted by additional molecular determinants which could be present in vivo. Therefore, significant discrepancies may arise from the two approaches. Apparently any remarkable difference in the readout is daunting. Indeed, if it occurs, it may be considered as an indication of a possible gap in the biological information and therefore this suggestion may be considered as useful. Taking into account these considerations, we may attempt to speculate on the comparison between the $K_d$ values obtained in vitro and the value derived from the in vivo study reported by Fujioka and coworkers. The observed difference in one order of magnitude among these $K_d$ may be due to the diverse activation state of MEK as well as the presence of additional factors regulating MEK-ERK interaction in vivo. Indeed, as reported by the group of Fujioka, only a fraction of MEK is activated in vivo, even when ERK activation is saturated, whereas all of the MEK is in the activated form in vitro. In addition, a number of evidence suggests the emerging role of scaffold proteins as regulators of MAPK signaling kinetics in terms of both potentiation and attenuation.22-24

It is well known that MEK-ERK interaction and signaling represents a complex signal module that may be controlled by different regulatory mechanisms. MEK is activated by phosphorylation of two Ser residues (Ser218 and Ser222 in MEK1; Ser222 and Ser226 in MEK2), and in turn binds and activates ERK by dual phosphorylation on Thr and Tyr residues (Thr202 and Tyr204 in ERK1; Thr185 and Tyr187 in ERK2). Moreover, inactive MEK can also bind (albeit weakly) to ERK and the phosphorylation of additional MEK amino acid residues have been reported in literature to have positive and negative effects on ERK phosphorylation and signaling.25-27 Therefore, one may speculate that the different phosphorylated residues play a role in regulating MEK-ERK binding affinity.

Interestingly, it is a controversial issue whether phosphorylation of ERK by MEK is a processive or distributive mechanism. In a processive catalysis, after binding the substrate, the kinase carries out two phosphorylation before releasing the final product; in contrast, in a distributive mechanism, the enzyme releases the intermediate monophosphorylated products, and a new collision is required for the conversion of this intermediate into the final product.28 However, a number of evidence demonstrates that dual phosphorylation of MAPKs occurs through a distributive two-collision mechanism.29-31 In this case, the $K_d$ values that we calculated from our in vitro measurements would represent the $K_d$ corresponding to MEK-ERK complex formation that precedes the first step of the distributive mechanism of catalysis. Interestingly, the obtained $K_d$ value is in excellent agreement with the corresponding value (50 nM) that is possible to calculate from the $k_{on}$ and $k_{off}$ reported by Markevich et al. 28 They developed a computational model of MAPK signaling based on parameter values retrieved from previously reported experimental data on the time course of MEK-catalyzed dual phosphorylation of ERK. For a future detailed characterization of MEK-ERK binding in this context, it will be interesting to analyze the interaction between MEK and monophosphorylated ERK using the proposed stopped-flow approach and compare the derived data with those reported in the case of MEK binding to unphosphorylated ERK.

A recent study by Baccarini et al. reports that MEK1-MEK2 heterodimers exist in the cells, and that they coexist with MEK1 and MEK2 monomers, with higher order complexes and possibly with homodimers.32 In this context, the role of heterodimer formation has been shown to be essential for ERK-mediated phosphorylation of Thr292 of MEK1, that attenuates MEK/ERK signaling through a negative feedback mechanism.32

Conclusions

Through the case study of MEK-ERK binding, we propose the stopped-flow approach as a suitable in vitro method to experimentally measure the kinetic parameters of binary protein-protein binding in a well-characterized and not time-consuming manner. This is of particular importance in the development of signal transduction mathematical models, in which the systems biologists are often faced with the difficulties concerning the choice of the kinetic constant values to be used in the model of interest. A more intensive use of stopped-flow fluorescence intensity technique may facilitate the generation of high-quality in vitro data. Having established a framework for deriving such valuable data, the natural extension of our work may consist in the kinetic analysis from a biological standpoint more closely to the real complex systems of MAPK cascade. This may be achieved by investigating the in vitro MEK-ERK interaction considering homodimer vs heterodimer formation as well as the presence/absence of specific phosphorylated amino acid residues in the recombinant proteins of interest.

Finally, the stopped-flow approach that we proposed may be applied in the context of binary protein-protein interaction belonging to other signaling cascades and contribute to a better comparison between in vitro and in vivo protein interaction behaviors, leading to an improvement in the development of signal transduction models for a fine elucidation of cell signaling mechanisms.
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Notes and references