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

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MEK and ERK are central components of the mitogen-activated protein kinase pathway. However, an accurate interaction has never been studied and accurate binding constants of the binary interaction have never been directly measured. In the present work, we studied the interaction between MEK and ERK by stopped-flow fluorescence intensity and evaluated the association and dissociation rate constants (k_{on} and k_{off}) from the kinetic study. We compared the results obtained by using commercial and homemade protein productions. The dissociation binding constant (K_d) value determined for the binding of MEK to ERK is in good agreement with the values obtained from the analysis of the kinase enzymatic reaction of previous *in vitro* studies.

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 studies[5,6] or from *in vitro* studies of the kinase enzymatic reactions.[7-9] There is only one recent work[10] 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

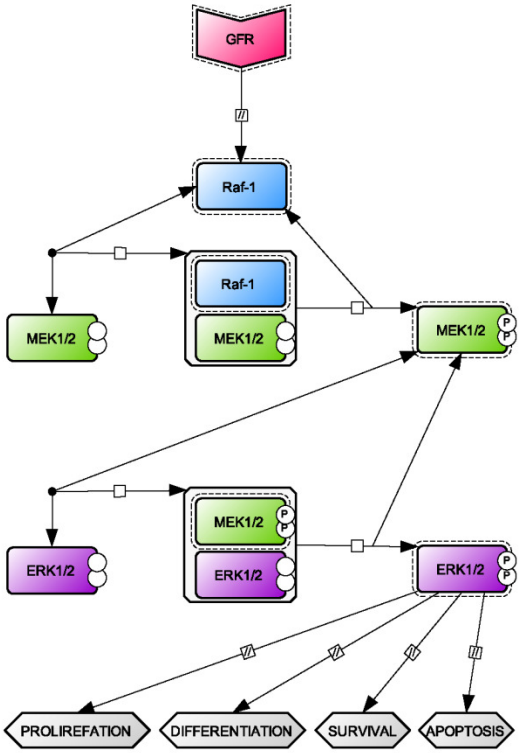

Fig. 1 Network topology of the mammalial MAP Kinases cascade: Raf/MEK/ERK axis. The signaling via this cascade, is usually initiated by growth factor receptor (GFR) activation, which transmit the signal by activating the MAPKKK, Raf-1. Active Raf-1 binds and activates the MAPKK, MEK (two isoforms, MEK1/2), by phoshorylation. Following a similar process, active MEK binds and activates the MAPK, ERK (two isoforms, ERK1/2). Active ERK regulates various cellular processes such as proliferation, differentiation, survival, and apoptosis. The network topology was realized using Cell Designer software according to Systems Biology Graphical Notation.[33]

intensity. The experimental procedure was based on the bioconjugation of ERK with a proper fluorophore, i.e. fluorescein-5-maleimide, followed by the analysis of its interaction with MEK by stopped-flow. This approach was applied to study the interaction of both commercial and homemade recombinant MEK and ERK proteins. Here kinetic values for the k_{off} and k_{on} of MEK-ERK binding have been experimentally measured for the first time avoiding their derivation from the analysis of the kinase enzymatic reaction. From the k_{off}/k_{on} ratio we calculated the corresponding K_d value that was compared with those obtained by previous studies.

2. Experimental Procedures

2.1 Reagents

The commercial recombinant MEK and ERK proteins used were full-length active MEK1 and full-length unactive ERK1. MEK1 was purchased from Upstate® as *N*-terminal GST and *C*-terminal 6His-tagged, expressed in *E. coli*, purified using glutathione-agarose followed by Ni^{2+}/NTA-agarose, activated using c-Raf and re-purified using Ni^{2+}/NTA-agarose; purity 98%; MW = 71 kDa. ERK1 was purchased from Upstate® as *N*-terminal Glutathione S-transferase (GST)-tagged protein, expressed in *E. coli* and 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 unactive 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;[15] 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, Southwestern) and Natalie Ahnn (University of Colorado, Boulder), respectively.

Fluorescein-5-maleimide was synthesised 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):

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

where A_{280} is the absorption of the conjugate at 280 nm; A_{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_{max} of the dye (c = 0.29); ε_{prot} (42230 cm^{-1} M^{-1}) and ε_{dye} (63096 cm^{-1} M^{-1}) are the molar extinction coefficients for the protein used and fluorescein-5-maleimide, respectively. The evaluation of c, $\varepsilon_{\mathrm{dye}}$ and $\varepsilon_{protein}$ 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.*[21] 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).

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

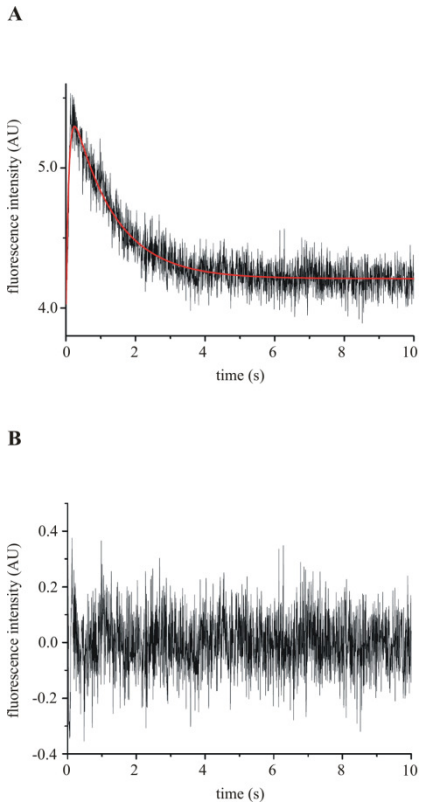

Fig. 2 (A) Stopped-flow fluorescence intensity record of the binding of 120 nM MEK to 10 nM ERK (commercial proteins). The solid thin line is the best fit to the data giving rate constants of 12.49 s^{-1}. (B) Residuals are also reported.

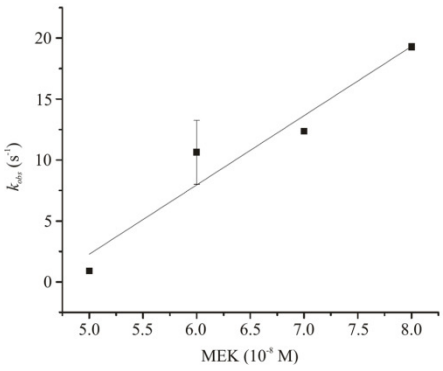

Fig. 3 Dependence of the k_{obs} determined by fluorescence intensity of the binding of ERK to increasing MEK concentrations (commercial proteins). Data points are the mean of five independent experiments ± standard deviation. Error bars not visible are concealed by the data point.

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 $5.49 \cdot 10^8$ $M^{-1}s^{-1}$. 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.

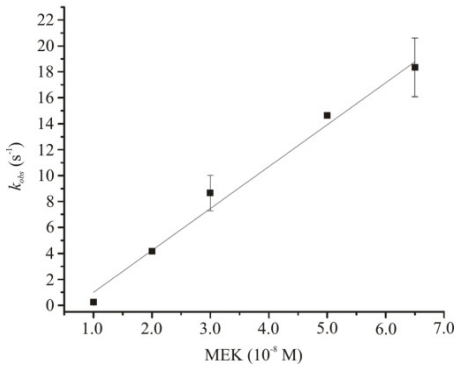

Fig. **4** Dependence of the k_{obs} determined by fluorescence intensity of the binding of ERK to increasing MEK concentrations (homemade proteins). Data points are the mean of five independent experiments ± standard deviation. Error bars not visible are concealed by the data point.

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_{obs} 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 $3.23 \cdot 10^8$ $M^{-1}s^{-1}$ 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.[21] Briefly, in the displacement experiment (see Scheme 1), a solution containing fluorescent ERK (ERK-fL) and MEK is mixed with an excess of non-fluorescent ERK (ERK-nfL). The concentration of ERK-fL is chosen to get a saturation of MEK, and then a high concentration of ERK-nfL is added so that ERK-fL dissociates from MEK and cannot reassociate. In this way the rate constant of the observed process is determined only by k_{-1}.

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).

Scheme 1 Displacement reaction.

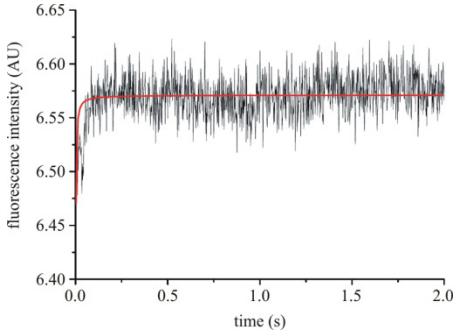

Fig. 5 Stopped-flow fluorescence record of a representative displacement experiment (homemade proteins): one syringe contained 50 nM biolabelled ERK, 50 nM MEK, and the other syringe contained 400 nM unlabelled ERK. The plot shows a fit to a single exponential with a rate constant of 20.5 s^{-1}. The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

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. The 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.[8,9] 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.[7-9] Compared to those obtained by *in vitro* methods, the K_d values derived from simulation modeling by the group of Schoeberl[5] and Yamada[7] 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 co-workers.[10] 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.

references	method	k_{on} (10^6 M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (nM)
This study	*in vitro*	323	18.7	58

Bhalla 2004[a,8], Sasagawa *et al.* 2005[a,9]	*in vitro*	16.2	0.6	37
Fujioka *et al.* 2006[10]	*in vivo*	0.88	0.088	100
Schoeberl *et al.* 2002[5]	simulation modeling	0.11	0.033	300
Yamada *et al.* 2004[6]	simulation modeling	0.318	0.9	2800

[a]Considering the Michaelis-Menten formulation as a special case of two reactions in sequence (Bhalla & Iyengar 1999), the values corresponding to K_m, k_{cat} and k_{off}/k_{cat} ratio (available in the DOQS database, http://doqcs.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,[10] 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 coesist 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 feed-back 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

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