Cell-free synthesis of the Ras-binding domain of c-Raf-1: binding studies to fluorescently labelled H-Ras

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Abstract It has previously been shown that the transient kinetics of the interaction between the Ras-binding domain of c-Raf-1 and the proto-oncoprotein Ras can be followed by stopped-flow measurements using the 2′,3′-(*N*-methylanthraniloyl) fluorescence of 2′,3′-(*N*-methylanthraniloyl) guanyl-5′-yl-imidodiphosphate-labelled Ras. In continuation of this work, we demonstrate that the His-tagged Ras-binding domain of c-Raf-1 can also be synthesized in a cell-free expression system. After purification by Ni^{2+} affinity chromatography, His-tagged Ras-binding domain of c-Raf-1 could be isolated in sufficient amounts for biochemical and biophysical investigations. The results obtained describe the first example of a cell-free synthesized protein which has been used for stopped-flow measurements to determine the transient kinetics of protein-protein interactions with an effector.

Key words: Cell-free expression; Transient kinetics; Ras-binding domain of c-Raf-1; H-Ras; Fluorescence

1. Introduction

The efficiency and range of applications of protein synthesis by cell-free expression has recently been considerably improved and the technique is now established as a versatile method with wide ranging applications in the biological sciences. A number of different in vitro systems have been developed which utilize a variety of cell extracts, such as those from *E. coli* or wheat germ. Furthermore, different experimental setups or protocols have been used to increase the yields [1–3]. On an analytical scale, in vitro expression or translation has been applied to, for example, the study of protein biosynthesis. On the other hand, examples of synthesis on a preparative scale using this technology are still quite rare. In this context, the development of continuous flow cell-free (CFCF) or semi-continuous systems is an important improvement [4,5]. Another interesting application is in vitro suppression, where proteins with artificial amino acids can be synthesized in a cell-free system [6]. However, the relative and absolute amounts of in vitro synthesized proteins in a batch system are still quite low when compared to bacterial expression systems, which leads to difficulties in their purification. Hence, there are only a few examples of cell-free synthesized proteins which have been purified for further biophysical investigations (e.g. [7–9]).

A general strategy to tackle the problem of yields in the μg scale is the use of an efficient purification by affinity chromatography, e.g. by using the strep-tag or the His-tag. In previous work, we have shown that the His-tagged Ras-binding domain (RBD/H) of c-Raf-1 can be purified by Ni^{2+} affinity chromatography to homogeneity [10]. Furthermore, as an important prerequisite for a meaningful analysis of the biochemical and biophysical properties, this C-terminal His-tag has only a negligible influence on the interaction with H-Ras [10]. Another important point is the experimental design of the biophysical investigations, which must be adjusted to the small amounts of the studied protein.

In this work, RBD/H was synthesized in a cell-free (*Escherichia coli*) batch system. Its binding to the proto-oncoprotein Ras was followed by stopped-flow measurements and the data have been fitted by numerical simulation to satisfy the lower than usual number of data sets. This example demonstrates that the cell-free synthesis of proteins can be used for the elucidation of biologically important questions such as the interaction of Ras with a downstream effector.

2. Materials and methods

The S30 extract was prepared from the *E. coli* strain D10 as described [11]. For the expression of RBD/H under the control of the T7 promoter, the encoding gene was cloned from the pET vector which was used for expression in *E. coli* [10] into the high copy plasmid pUCBM20 together with the T7 promoter region. For in vitro expression, the DNA template was linearized by restriction with *Aat*II, 470 bp downstream of the *rbd/h* gene.

Cell-free expression was optimized and performed as described [11] on a 30 μl or 1 ml scale. T7 RNA polymerase was prepared according to the published procedure [12]. The reaction mixture contained 4 μl 1 μg/μl *Aat*II-linearized DNA template in diethylpyrocarbonate-H_2O, 4 μl 0.1 M $Mg(OAc)_2$, 0.5 μl 10 mg/ml rifampicin in 10% (v/v) MeOH, 7.7 μl low molecular weight mix [11] (+1.5 μl pyruvate kinase (2 U/μl)+1 μl RNAse inhibitor in 100 μl), 1.4 μl protease inhibitor mix, 9 μl S30 extract, 2.4 μl 315 μM [^{14}C]leucine in 2% (v/v) EtOH (700 dpm/pmol), 1 μl T7 RNA polymerase (60 U/μl).

The amount of synthesized protein was determined after trichloroacetic acid precipitation by measuring the radioactivity of the incorporated [^{14}C]leucine in a scintillation counter. [^{14}C]leucine was obtained from ICN Biomedicals (Eschwege).

SDS-PAGE was performed as described [13] and fluorography according to [14]. The N-terminal sequence of the cell-free expressed RBD/H was determined by Edman degradation after blotting of the purified protein onto a polyvinylidene difluoride (PVDF) membrane. The protein concentration was determined using the Bradford assay [15].

The synthesized RBD/H was purified from the cell-free batch system by Ni^{2+} affinity chromatography using the Ni^{2+}-NTA spin kit from Qiagen (Hilden). The samples (1 ml) were centrifuged for 10 min at 8°C and filtered over a Low Binding Durapore membrane (Milli-

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Abbreviations: GppNHp, guanyl-5′-yl-imidodiphosphate; mant, 2′,3′-(*N*-methylanthraniloyl), a fluorophore attached at the 2′- or 3′-position; RBD, Ras-binding domain of c-Raf-1; RBD/H, RBD with an additional His-tag at the C-terminus; PVDF, polyvinylidene difluoride

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pore). The filtrate (3 ml) was loaded onto a Ni^{2+}-NTA spin column (3 ml/column) which was previously equilibrated with buffer A (300 mM NaCl, 50 mM phosphate, 5 mM imidazole, pH 8). The column was washed three times with buffer A and three times with buffer B (A with 20 mM imidazole). RBD/H was eluted by increasing the imidazole concentrations to 200 mM. In all steps, the columns were centrifuged at $150 \times g$ for about 2 min. The combined fractions were concentrated with centrifugal concentrators from Filtron Technology and used directly for the stopped-flow measurements.

Stopped-flow measurements, numerical simulation and fitting of the data sets to a kinetic model were performed as described [10].

3. Results and discussion

The cell-free synthesis of RBD/H was performed in a batch system with S30 extracts from *E. coli*. For the expression of the *rbd/h* gene, we used the T7 promoter which has already been widely used for selective expression in bacterial cell-free systems [16,17]. The subcloning of the encoding gene of the RBD/H into the high-copy plasmid pUCBM20 provided high yields of the required plasmid. The additional His-tag at the C-terminus allows the subsequent purification of the synthesized protein by Ni^{2+} affinity chromatography.

Finally, an expression yield of 40 μg/ml was obtained. Analysis by fluorography showed that only one protein with a molecular weight in the range expected for RBD/H was synthesized (Fig. 1, lane 2). Its broad band is due to the high protein concentration of the sample. The positions of the marker bands and that of the authentic RBD/H have been indicated by bars.

In the next step, RBD/H was purified by Ni^{2+} affinity chromatography. For small amounts of a His-tagged protein, the spin kit system from Qiagen is especially suited, since the spin columns have a lower binding capacity compared to Ni^{2+}-NTA agarose and they are easy to handle. After centrifugation and filtration of the cell-free expression samples, a total of 3 ml was loaded onto one column. After washing with 5 and 20 mM imidazole, the RBD/H was eluted with 200 mM imidazole. Fig. 2 shows the SDS-PAGE analysis of the purification steps. The protein bands were visualized by Coomassie blue staining. Most of the impurities could be eluted with buffer A (Fig. 2, lane 4). Increasing the imidazole concentration to 20 mM in the wash buffer (buffer B) only led to the elution of minor impurities in the higher molecular weight range (Fig. 2, lane 5). Lane 6 and 7 show purified RBD/H from two different Ni^{2+}-NTA spin columns. As can be seen, only minor impurities (mostly in the high molecular weight range) contaminate the RBD/H samples. From 3 ml cell-free extracts, about 90 μg RBD/H (75% recovered yield based on synthesized protein) could be obtained, which was sufficient for the subsequent biophysical analysis.

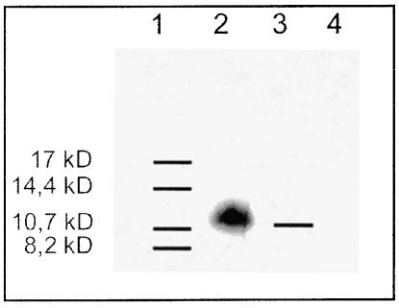

Fig. 1. Analysis of an optimized cell-free synthesis of RBD/H by fluorography. Lane 1: bars indicate the positions of the PMW marker bands on the corresponding polyacrylamide gel, lane 2: 5 μl of a cell-free expression sample, incubated at 37°C for 1 h with L-[^{14}C]leucin, lane 3: the single bar indicates the position of the RBD/H band expressed in *E. coli* as a reference on the corresponding polyacrylamide gel, lane 4: negative control (cell-free expression sample without T7 RNA polymerase)

The purified protein was analyzed by SDS-PAGE, blotted onto a PVDF membrane and N-terminally sequenced by Edman degradation. The determined sequence (DPSKT) is in accordance with the N-terminal sequence of the RBD. Finally, the protein samples were concentrated and used directly for stopped-flow measurements using the conditions previously described [10].

In these experiments, we used H-Ras loaded with the fluorescent, non-hydrolyzable GTP analogue 2′,3′-(*N*-methylanthraniloyl) (mant) guanyl-5′-yl-imidodiphosphate (GppNHp). Thus, the interaction of RBD/H with this complex can be followed by a change of the mant fluorescence. The kinetics of this process are in the ms–s time range and could thus be investigated by the stopped-flow method. However, the small amount of the cell-free expressed RBD/H available for these experiments did not allow a variation of the concentration under pseudo-first order conditions. Therefore, a different approach for the determination of kinetic parameters was chosen.

Assuming the simplest model (one step binding: Scheme 1), the differential equations were integrated numerically and constants were fitted to this model using two different data sets obtained at different RBD/H concentrations. This was performed with the program Scientist and a single set of constants was obtained which were compatible with both data sets. The model equation file for the simultaneous fit of two fluorescence transients is presented in Scheme 2.

$$\text{RBD} + \text{Ras.mantGppNHp} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{RBD.Ras.mant}^{*}\text{GppNHp}$$

Scheme 1.

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// second order, equilibrium
IndVars: t
DepVars: C, C1, F, F1
Params: k1, k2, Ya, Yc, A0, B0
C'=k1*(A0-C)*(B0-C)-k2*C
F=(A0-C)*Ya+C*Yc
C1'=k1*(A0-C1)*(B0*2-C1)-k2*C1
F1=(A0-C1)*Ya+C1*Yc
//Initial conditions
t=0
C=0
C1=0
***
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Scheme 2.

C and *C1* are the concentrations of the H-Ras.mant-GppNHp.RBD/H complex in the two experiments with the unit μM, *F* and *F1* are the observed fluorescence signals (photomultiplier output in volts), k_1 and k_2 are the rate constants for the forward (k_1) and reverse reactions (k_2) with the

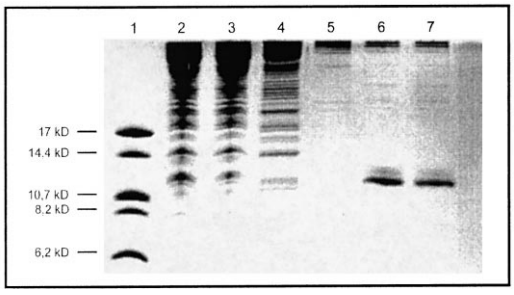

Fig. 2. SDS-PAGE analysis of the RBD/H purification synthesized in the cell-free expression system. Lane 1: PMW marker, lane 2: 5 μl of a cell-free expression sample, incubated at 37°C for 1 h, lane 3: 5 μl flow-through of the Ni^{2+}-NTA spin column, lane 4: 50 μl of the washing pool of buffer A, lane 5: 50 μl of the washing pool of buffer B, lane 6 and 7: elution pools of two different Ni^{2+}-NTA spin columns. All samples were precipitated with acetone and redissolved in sample buffer.

units μM/s and s^{-1}, respectively, Y_a and Y_c are the fluorescence yields with the units V/μM and A_0 and B_0 are the initial concentrations of H-Ras.mantGppNHp and RBD/H, respectively, also with the unit μM. C' and $C1'$ stand for dC/dt and d$C1$/dt.

The concentrations of proteins were allowed to float during the fit procedure to determine the concentration of active cell-free synthesized protein (Scheme 2).

In our first experiment, we obtained two data sets for the interaction of 0.5 μM H-Ras.mantGppNHp with different concentrations (0.5 and 1 μM) of RBD/H expressed in *E. coli* to verify this approach to the determination of the kinetic parameters by comparison with the previously determined values of the *E. coli*-expressed protein (Fig. 3a). The kinetic parameters for the interaction of the two proteins which result from the numerical simulation are in very good agreement with the previously obtained data from the pseudo-first order measurements and give a dissociation constant of 0.3 μM [10]:

$k_1 = 2.3 \times 10^7$ M^{-1}/s	$Y_a = 1.93$ V/μM	$A_0 = 0.51$ μM
$k_2 = 6.8$ s^{-1}	$Y_c = 1.77$ V/μM	$B_0 = 0.47$ μM

where A is H-Ras.mantGppNHp, B is RBD/H, Y_a is the fluorescence yield of H-Ras.mantGppNHp and Y_c is the fluorescence yield of the complex.

Furthermore, the concentrations which were determined by the simulation correlate very well with the concentrations determined by a protein assay (0.5 μM for H-Ras.mantGppNHp, 0.5 and 1 μM for RBD/H).

The fit for the interaction of 0.5 μM H-Ras.mantGppNHp with different concentrations (0.58 and 1.16 μM) of the cell-free synthesized RBD/H is shown in Fig. 3b.

The numerical simulation with the model presented in Scheme 2 gave the following results for the parameters:

$k_1 = 1.7 \times 10^7$ M^{-1}/s	$Y_a = 2.07$ V/μM	$A_0 = 0.48$ μM
$k_2 = 5.9$ s^{-1}	$Y_c = 1.94$ V/μM	$B_0 = 0.50$ μM

The rate constants appear to be slightly reduced compared to the RBD/H expressed in *E. coli* but they are still in the same range, i.e. ca. 2×10^7 M^{-1}/s for the association rate constant and ca. 6 s^{-1} for the dissociation rate constant.

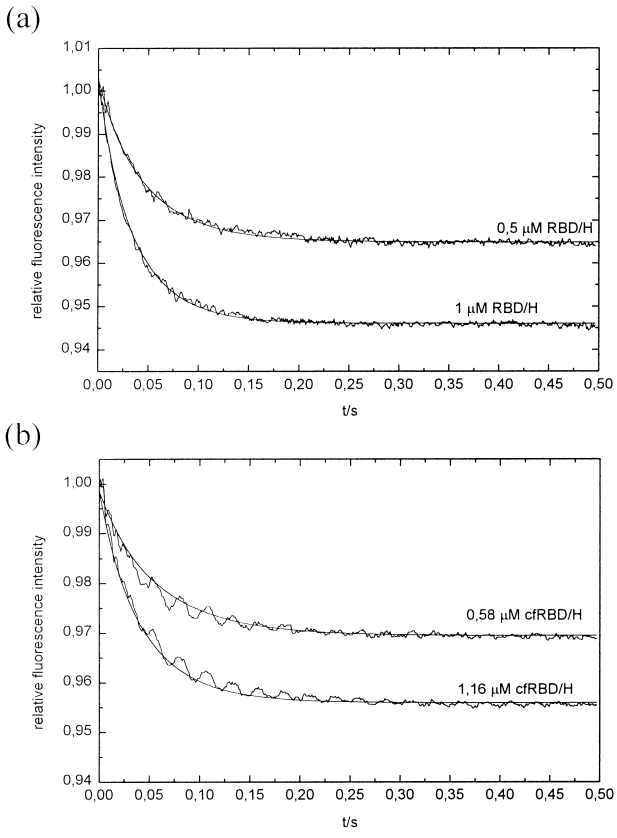

Fig. 3. (a) Simultaneous fit of the fluorescence transients using the described model equation file (scheme 2) with the program Scientist for the RBD/H expressed in *E. coli*. 0.5 μM H.Ras.mantGppNHp was rapidly mixed with 0.5 μM and 1 μM RBD/H at 25°C. The excitation wavelength was 366 nm with detection through a 389 nm cut-off filter. (b) Simultaneous fit of the fluorescence transients using the described model with the program Scientist for the cell-free synthesized RBD/H. The same conditions as described above with 0.58 μM and 1.16 μM cell-free synthesized RBD/H.

The determined dissociation constant for the ternary complex is 0.34 μM, which is also in good agreement with the K_d value for the RBD/H expressed in *E. coli*.

Another aspect is also very important in this case. The simultaneous simulation of the data sets allows determination of the effective concentration (0.5 μM) of the RBD/H, which is about 15% lower for the cell-free synthesized protein than determined by a protein assay (0.58 μM). This correlates with the SDS-PAGE analysis which shows some impurities in the elution sample of the cell-free synthesized RBD/H. If these contaminations are taken into consideration, the cell-free synthesized protein has about the same degree of activity as the RBD/H expressed in *E. coli*.

Our work presents an illustrative example that cell-free synthesized proteins are now readily amenable for biochemical and biophysical studies like stopped-flow techniques. However, an efficient purification scheme, such as affinity chromatography, and special approaches for dealing with small amounts of protein are still mandatory. This procedure can certainly be extended to the analysis of protein-protein interactions with fluorescently labelled proteins which have been synthesized by in vitro suppression.

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