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Affinity of the monoclonal antibody M1 directed against the FLAG peptide

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Abstract

The FLAG (Sigma, St. Louis, MO, USA) peptide is a frequently used hydrophilic and immunogenic fusion tag which was specifically designed to facilitate rapid purification by immunoaffinity chromatography. The monoclonal antibody M1 recognizes the free N-terminus of the peptide tag in a calcium dependent manner. Dissociation of the complex can be performed by the addition of chelating agents such as EDTA. This effect can be exploited for immunoaffinity purification of FLAG-tagged fusion proteins. Kinetic information obtained from monitoring interactions in real-time measurement (Biacore 2000) using surface plasmon resonance as detection principle did not show any difference for association and dissociation rate constants in the presence ($k_a=3.03\cdot10^3\ M^{-1}\ s^{-1}$ $k_d=1.25\cdot10^{-3}\ s^{-1}$) and in the absence of Ca^{2+} ($k_a=3.59\cdot10^3\ M^{-1}\ s^{-1}$, $k_d=1.16\cdot10^{-3}\ s^{-1}$). These findings corroborate the reports from *Mol. Immunol.* 33 (1996) 601–608 describing similar binding analyzed by enzyme-linked immunosorbent assay experiments. These investigations are in contrast to the observations in immunoaffinity chromatography with immobilized anti-FLAG antibody M1.

Keywords: Kinetic analysis; Rate constants; Monoclonal antibodies; Peptides; FLAG peptide

1. Introduction

The FLAG (Sigma, St. Louis, MO, USA) peptide is a frequently applied hydrophilic and immunogenic fusion tag which was specifically designed to facilitate rapid purification by immunoaffinity chromatography. The peptide consists of eight amino acids (DYKDDDDK) [1]. Due to this small size, the marker peptide can be encoded by a single synthetic oligonucleotide. The last five amino acids (DDDDK) represent the minimal enterokinase specifity site, thus enabling the proteolytic removal of the tag. Depending on the application purpose the FLAG peptide can be fused either to the N- or C-terminus of a given fusion protein. The free N-terminus of the FLAG peptide can be recognized by a monoclonal antibody; the anti-FLAG antibody M1. This antibody forms a complex with the FLAG peptide in a calcium dependent manner which can be dissociated by chelating agents such as EDTA [2]. Although many applications of the FLAG peptide especially in immunoaffinity chromatography are reported [1–3] there are no exact data available on k_a and k_d values and of the thermodynamical properties of anti-FLAG antibody M1.

Conventional methods for kinetic analysis such as equilibrium dialysis, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and others can give valuable information on the conditions and specificity of an interaction but are

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unable to follow a process over the time. Surface plasmon resonance (SPR) biosensors have become an established tool for analyzing protein–protein interaction in real-time. Biomolecular interaction analysis (BIA) technology has been used to great extend for a variety of different applications, primarily to characterize protein–protein interactions, such as antibody–antigen [4–8] and receptor–ligand interactions [9–11]. BIA technology has also been used to study growth factors [12], signal transduction [13–15] and immune regulation [16,17]. We used this system to monitor the kinetics of interaction between a commonly used peptide tag in affinity chromatography, the so-called FLAG peptide and a monoclonal antibody, anti-FLAG M1, which is directed against the free N-terminus of the FLAG peptide.

2. Theory

Real-time BIA exploits the optical phenomenon SPR to monitor biomolecular interactions. In an optical biosensor experiment, one of the interacting components (the ligand, B) is immobilized on the surface of a sensor chip whereas the other (the analyte, A) is in free solution over the sensor surface. The formation and dissociation of the complex (AB) are controlled by the respective association (k_a) and dissociation (k_d) rate constants. Assuming pseudo-first order kinetics:

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB \tag{1}$$

the rate of complex formation during analyte injection is given by:

$$\frac{dR}{dt} = k_a C R_{max} - (k_a C + k_d R_t) \tag{2}$$

where dR/dt is the rate of change of the SPR signal, k_a is the association constant, k_d the dissociation rate constant, C is the analyte concentration, R_{max} the maximum analyte binding capacity in response units (RU), and R_t is the signal at time t. The integral form of Eq. (2), expanded by the R_i the background binding in response units (RU):

$$R_t = \left(\frac{C k_a R_{max} \{1 - e^{-[(C k_a + k_d)t]}\}}{C k_a + k_d} \right) + R_i \tag{3}$$

has been used to fit the response curve during the association phase. From these fits k_a (M^{-1} s^{-1}), k_d (s^{-1}) and K_A (M^{-1}) can be estimated. As the interaction proceeds, the concentration of analyte in the surface layer changes, giving an SPR response which can be followed in real-time. Detection depends on alterations in the mass concentration of analyte at the biospecific interface and does not require any labeling of the analyte. In contrast to other conventional methods where detection is restricted to a definite time point, BIA provides monitoring of all stages in an interaction sequence in real-time.

The rate of dissociation can be expressed by:

$$\frac{dR}{dt} = -k_d R_t \tag{4}$$

and the integrated form:

$$R_t = (R_0 e^{-k_d t}) + R_{(t-\infty)} \tag{5}$$

can be used to fit response curves during the dissociation phase. Here R_0 is the response at the beginning of the dissociation phase and $R_{(t-\infty)}$ is the response at infinite time accounting for the background signal. The dissociation constant can be estimated from this fit. At equilibrium, association and dissociation rates are equal and the equilibrium binding constant given by:

$$K_A = \frac{1}{K_D} = \frac{k_a}{k_d} \tag{6}$$

According to O'Shannessy and Winzor [18], the value for k_d obtained from first-order approximation of a time-span of the dissociation phase can be inserted into the fitting equation for the adsorption phase. The resulting values for k_a and R_{max} show higher confidence. Additionally, injection and analysis of different analyte concentrations can reveal deviations from first-order kinetics and limitations due to mass transfer problems [6].

There are three major methods used to calculate kinetic rate constants from biosensor data: linearization [19], curve fitting with analytical integration [20], and curve fitting with numerical integration

[21]. In our case experimental sensorgram data were analyzed assuming a pseudo-first order reaction kinetic.

3. Experimental

3.1. Model protein

Green fluorescent protein (GFP) from *Aequoria victoria*, the jellyfish was chosen as fusion partner to the FLAG peptide. The cDNA encoding for the FLAG–GFP fusion protein was cloned into an appropriate yeast expression vector and subsequently transformed into *Saccharomyces cerevisiae*. Yeast cells were grown on yeast peptone high stability medium (YPHSM) at 30°C. Protein expression was induced by the addition of 10 m*M* $CuSO_4$ at an $A_{600nm} = 0.5$. Eight hours post induction cells were harvested by centrifugation. To release the expressed fusion protein cell pellets were incubated in a sorbitol buffer supplemented with Lyticase (Sigma, Munich, Germany) inducing spheroplast formation. Cell membrane degradation was monitored at an $A_{800\ nm}$. Finally cells were subjected to a hypotone shock to release the fusion protein. The clarified supernatant was subjected to further purification by combined ion-exchange chromatography and size-exclusion chromatography in a continuous annular chromatograph as described by Uretschläger and Jungbauer [22].

3.2. Immobilization of antibody M1 on CM5 sensor chip

Sensor Chip CM5, HBS running buffer [10 m*M* 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid (HEPES), 150 m*M* NaCl, 0.005% surfactant P20, supplemented with either 5 m*M* $CaCl_2$ or 10 m*M* EDTA, pH 7.4] and an amine coupling kit [*N*-ethyl-*N′*-(dimethylaminopropyl)carbodiimide (EDC), *N*-hydrosysuccinimide (NHS) and ethanolamine hydrochloride, pH 8.5] were purchased from Biacore (Uppsala, Sweden). Anti-FLAG antibody M1 (Sigma) was immobilized using the standard amine-coupling procedure [23]. The sensor surface was activated with a EDC/NHS pulse. The ligand (anti-FLAG antibody M1), dissolved in 10 m*M* sodium acetate buffer, pH 4.5 was then subjected to the activated dextran layer. For deactivation of the surface 1.0 *M* ethanolamine was injected. The recommended immobilization level of ligand in kinetic analysis should be in the range of 100–1000 RU. For evaluation of the affinities of anti-FLAG antibody M1 the immobilization level for the ligand were 446.4 RU. The unit RU is proportional to the surface concentration, whereas 1000 RU correspond to approximately 1 ng/mm^2 [24].

4. Results and discussion

To date no exact data on association and dissociation constants of whole anti-FLAG antibody M1 are available. To sustain reliable k_a and k_d values for anti-FLAG antibody M1 two concentration series of purified FLAG–GFP fusion protein were performed using CM5 sensor chip with immobilized whole anti-FLAG antibody M1. Rate constants were evaluated under so-called binding and non-binding conditions of the anti-FLAG antibody M1 to the FLAG peptide. In immunoaffinity chromatography with immobilized M1 antibody binding of FLAG fusion proteins is accomplished in the presence of Ca^{2+} and elution is effected with EDTA to remove Ca^{2+}. Therefore the FLAG–GFP fusion protein was subjected to HBS running buffer supplemented with either 5 m*M* $CaCl_2$ to enable complex formation of the antibody and the FLAG peptide or with 10 m*M* EDTA to inhibit complex formation. Subsequently samples were diluted at ratios of 1:2, 1:3, 1:5, 1:10 and 1:100. Kinetic analysis experiments were performed by starting from the undiluted sample to the sample of lowest fusion protein concentration. As a blank a run with just HBS buffer alone was also included into the experimental measurements. All experiments were carried out at 25°C. Sensorgrams for binding of FLAG–GFP fusion protein to anti-FLAG antibody M1 in the presence and absence of Ca^{2+} ions are shown in Figs. 1 and 2. Similar response curves were observed in the presence and absence of Ca^{2+}, although differences were expected from observations made in immunoaffinity chromatography. Thereby FLAG-tagged proteins bind in the presence of Ca^{2+} and elution is effected by chelating agents such as EDTA [1–3,25]. Mass transfer limita-

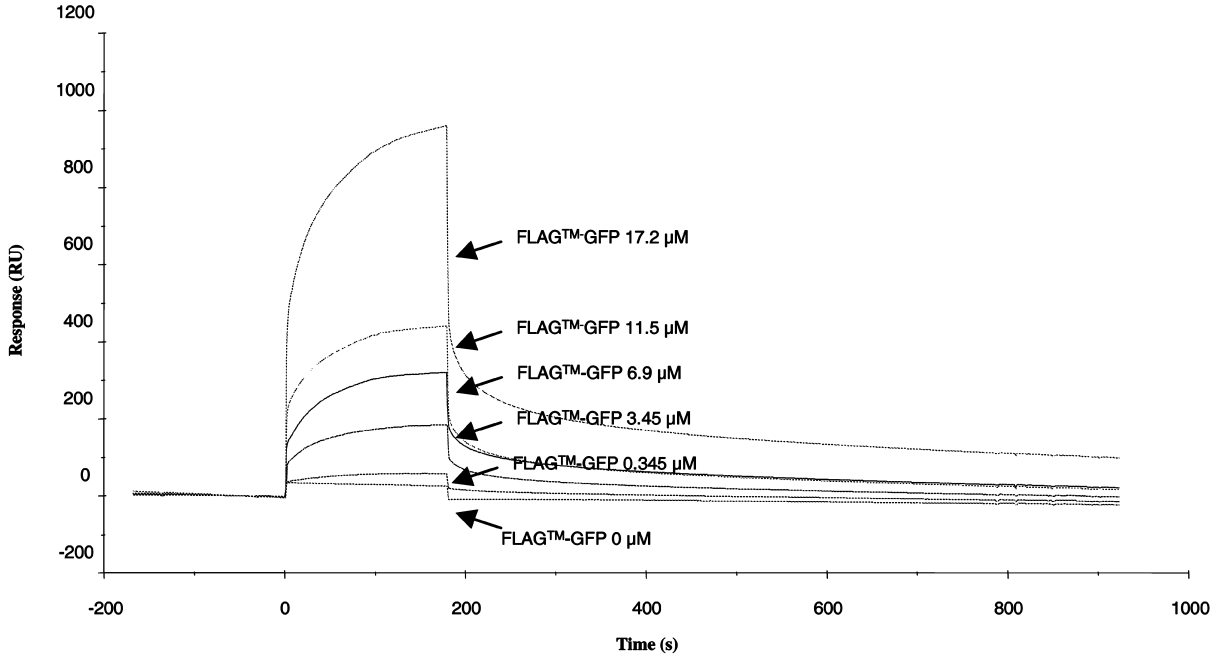

Fig. 1. Sensorgram of FLAG–GFP fusion protein in the presence of Ca^{2+}. A 90-µl volume of sample was injected with a flow-rate of 30 µl/min followed by injection of HBS buffer supplemented with 5 m*M* Ca^{2+}.

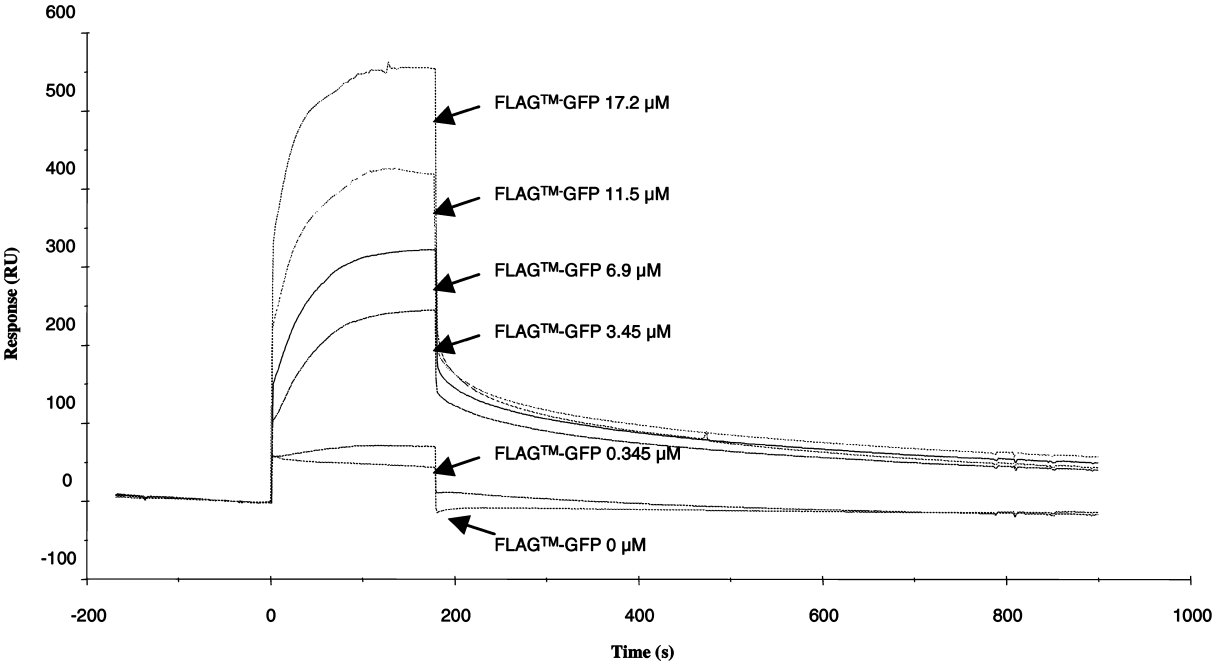

Fig. 2. Sensorgram of FLAG–GFP fusion protein in the absence of Ca^{2+}. A 90-µl volume of sample was injected with a flow-rate of 30 µl/min followed by injection of HBS buffer supplemented with 10 m*M* EDTA.

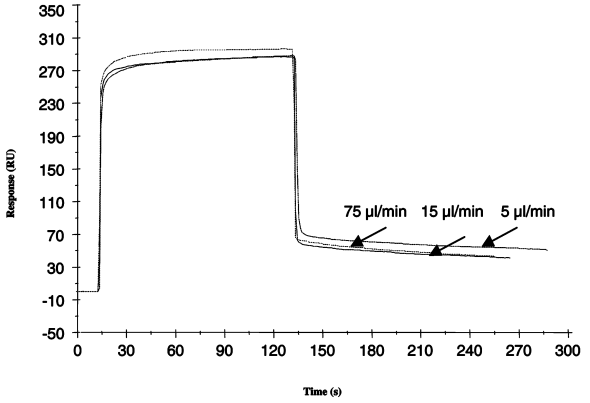

Fig. 3. Sensorgram of FLAG–GFP fusion protein mass transfer control experiments. FLAG–GFP fusion protein (6.9 μ*M*) was injected at three different flow-rates (5 μl/min, 15 μl/min, 75 μl/min) for 2 min each.

tions have been made responsible for non-reliable kinetic data measured with Biacore 2000. To evaluate possible mass transport limitations a defined concentration of the model protein was analyzed under three different flow-rates. The obtained sensorgrams were superimposed. The identical response curves of three different flow-rates indicated that a significant mass transfer limitation does not interfere with our kinetic analysis (Fig. 3).

Experimental data were fitted using Eqs. (3) and (4). BIA evaluation software version 3.1 was applied according to the procedures described in the software manual. The dissociation and association rate constants (k_d, k_a) were determined and association equilibrium constant (K_A) was calculated using Eq. (6). K_D, the dissociation equilibrium constant, is often used for convenience since it is in molar units. The kinetics for binding of the FLAG peptide to anti-FLAG antibody M1 are summarized in Table 1.

Our findings for whole antibody M1 corroborate the data reported by Hopp et al., using an ELISA, where plates were coated with a FLAG peptide–ovalbumin conjugate and a calcium dependent binding of the whole anti-FLAG antibody M1 as well as of its Fab fragments was tested [26]. They did not observe a different binding behavior with or without Ca^{2+} for whole anti-FLAG antibody M1. They interpret their results due to the bivalent nature of the whole antibody since the monovalent Fab fragment of M1 showed a Ca^{2+} dependent binding behavior in ELISA experiments.

As a possible explanation for the elution behavior an enthalpy (H) entropy (S) compensation could serve. The Gibbs free energy (ΔG) does not change with temperature although the contribution of enthalpy change (ΔH) and entropy change ($T\Delta S$) are significant. They compensate each other and therefore ΔG does not change. This implies that the equilibrium binding constant remains constant, since ΔG can be expressed as $\Delta H - T\Delta S$. This has been also reported for other antibodies [27]. Upon Ca^{2+} binding entropy/enthalpy compensation might also take place. We think that with the addition of the chelating agent a transition state is formed, which has less affinity compared to the equilibrium state in the presence or absence of Ca^{2+}. This transition state cannot be analyzed with the applied method. From thermodynamics (K_A, K_D) elution in affinity chromatography with anti-FLAG antibody M1 should not be possible, which contradicts with experimental data. These observations have to be subjected to further analysis.

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Table 1
Kinetic constants of anti-FLAG antibody M1 in the presence and absence of Ca^{2+} ions

Ca^{2+}/EDTA	k_a (1/*M* s)	k_d (1/s)	K_A (1/*M*)	K_D (*M*)
5 m*M* Ca^{2+}	$3.03 \cdot 10^{3}$	$1.25 \cdot 10^{-3}$	$2.42 \cdot 10^{6}$	$4.12 \cdot 10^{-7}$
10 m*M* EDTA	$3.59 \cdot 10^{3}$	$1.16 \cdot 10^{-3}$	$3.09 \cdot 10^{6}$	$3.24 \cdot 10^{-7}$

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