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Complex formation of a calcium-dependent antibody: A thermodynamical consideration

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Abstract

The elution of FLAG-fusions (an octapeptide with the sequence DYKDDDDK) from immobilized anti-FLAG antibody M1 cannot be explained by a switch of the equilibrium binding constant to a lower value. To get a further insight into thermodynamics, the binding of anti-FLAG antibody M1 to the FLAG peptide was studied by real-time biosensor technology at seven different temperatures in the range from 5 to 35 °C. Binding studies were performed in the presence and absence of calcium. Thermodynamic parameters such as change in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) were evaluated from the corresponding equilibrium data applying the integrated Van't Hoff equation. In contrast to similar kinetic data obtained, the contribution of ΔH and ΔS to ΔG in the presence or absence of calcium results in a different conformation of the antibody–antigen complex under binding and non-binding conditions. Therefore, complex dissociation with EDTA must be effected during a transition state of complex formation and dissociation.

Keywords: Kinetic studies; Thermodynamic parameters; Biosensers; Antibodies; Peptides; Proteins

1. Introduction

Fusion protein-technology became a common tool for rapid expression, purification and detection of recombinant proteins [1–6]. The so-called FLAG system represents such a tool whereas the FLAG tag (an octapeptide with the sequence DYKDDDDK), features a high selectivity upon interaction with its counterpart, a monoclonal antibody, in a Ca^{2+}-dependent manner [7]. According to the nature of application purpose, the FLAG peptide can be N- or C-terminally fused to a fusion protein of choice. Three monoclonal antibodies (M1, M2, M5) are available for specific peptide detection [8–11]. M2 and M5 recognize an internal sequence of the octapeptide whereas M1 is directed against the free N-terminus of the peptide tag. In immunoaffinity chromatography complexation of anti-FLAG antibody M1 and the FLAG peptide is calcium-dependent. Elution can be effected by the addition of chelating agents such as EDTA. At least a few hundred papers have reported on the outstanding properties of the FLAG system. Contrary to reports found in the literature, in our experiments the gained elution profile in immunoaffinity chromatography with anti-FLAG antibody M1 is characterized by intense tailing [12]. Also, enzyme-linked immunosorbent assay (ELISA) experiments showed a calcium-independent binding of the FLAG tag to the whole antibody M1 [13]. To clarify these apparently

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contradictory results, the characterization of the affinities and rate constants of the complex formation of the FLAG tag and the anti-FLAG antibody M1 are therefore of fundamental interest. In addition, the determination and understanding of the thermodynamics of protein–protein interactions are a common goal and have great impact on practical applications such as the characterization of the structure–function relationship of biomolecules. To date, a wide variety of techniques have been used to study such interactions. To obtain complex structural information, high-resolution X-ray diffraction studies, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are used. The limitations of NMR and MS are due to the fact that the resolution for large complexes or proteins is not sufficient [14]. Functional analysis can be obtained in terms of specificity by kinetic rate constants and equilibrium constants as well as by the thermodynamic parameters of the interaction. Concentration assays can be used to qualify equilibrium constants of strong affinity interactions; ELISA is the most widely used. For thermodynamical analysis, microcalorimetry and real-time binding assay based on surface plasmon resonance (SPR) are the methods of choice [15–19]. Additionally, these techniques have significant implications on the development and understanding of potential ligands and their binding behavior in affinity chromatography.

We have recently obtained kinetic information from monitoring the interaction of anti-FLAG antibody M1 and the FLAG peptide in real-time measurement (BIACORE 2000) using surface plasmon resonance as detection principle. The results coincide with previously performed ELISA experiments. From thermodynamics (equilibrium constants K_A/K_D), elution in affinity chromatography with anti-FLAG antibody M1 should not be possible, which contradicts with experimental data in immunoaffinity chromatography [20]. Therefore, supplementary investigations in terms of thermodynamical properties of this antibody–antigen interaction are of great demand and interest.

The aim of this paper was to describe the thermodynamic background of biomolecular interactions via kinetic data obtained by BIACORE experiments. Binding curves were obtained in the temperature range from 5 to 35 °C. The rate and equilibrium constants were calculated at each temperature. Equilibrium data were used to calculate the contribution of ΔH and ΔS from integrated Van't Hoff plots.

2. Experimental

2.1. Fusion protein and monoclonal antibody M1

As fusion partner to the FLAG peptide, green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was chosen. The respective cDNA of the fusion protein was cloned and expressed in *Saccharomyces cerevisiae* [21]. Recombinant fusion protein was extracted and further purified as described by Uretschläger and Jungbauer [22]. To avoid interference of autofluorescence from GFP with the resulting SPR signal in Biacore, fluorescence was quenched by the addition of 16% HCl. Subsequently the pH of the sample was adjusted to 7.4 by desalting over a Sephadex C-25 column. Monoclonal anti-FLAG antibody M1 was purchased from Sigma, St. Louis, MO.

2.2. Preparation of sensor surface

The BIACORE 2000 system as well as the reagents for interaction analysis including sensor chips 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*-hydroxysuccinimide (NHS) and ethanolamine hydrochloride, pH 8.5] were purchased from Biacore (Uppsala, Sweden). Anti-FLAG antibody M1 was immobilized using the standard amine coupling procedure [23]. The dextran layer of the sensor surface was activated with an EDC/NHS pulse. Anti-FLAG antibody M1, dissolved in 10 m*M* sodium acetate buffer, pH 4.5 was then subjected to the activated dextran layer. Subsequently, deactivation of the surface was accomplished by the injection of 1.0 *M* ethanolamine. Immobilization levels of ligand in kinetic analysis should be in the range of 100–1000 response units (RU). For our studies, immobilization levels of anti-FLAG M1 were 401 RU. The unit RU is proportional to the surface

concentration, whereas 1000 RU correspond to approximately 1 ng/mm^2 [24].

2.3. Kinetic measurements and data analysis

The interaction of anti-FLAG antibody M1 with the FLAG peptide was investigated at seven temperatures over the range 5–35 °C (5 °C step) in the presence and absence of calcium, respectively. Each temperature analysis was done at least in duplicate. A summary of the experimental conditions is shown in Table 1.

Experimental data were obtained in the form of sensorgrams representing the change in SPR response as a function of time. CLAMP, a shareware software (http://www.cores.utah.edu/interaction) [25] was used to fit the data according to a two-step conformational change model. The software combines numerical integration and non-linear curve-fitting routines. It is designed to interpret complex interactions recorded on biosensors by simultaneously analyzing association and dissociation phase data. The input data, a reaction model, and reasonable starting values for the unknown parameters are loaded. The program generates a series of differential rate equations based on the model and integrates them numerically to simulate a set of response curves using a semi-implicit extrapolation method. This method is capable of handling stiff sets of differential rate equations. Simulated data are then subtracted from experimental data in order to calculate the χ^2 value. The initial estimates for the rate constants are then adjusted to minimize a goodness of fit merit function usually referred to χ^2 using a Levenburg–Marquadt non-linear minimization algorithm [26]. The simulation and minimization process is repeated until the change in the χ^2 is less than 0.1%. From the resulting association rate constants (k_a) and dissociation rate constants (k_d) the association equilibrium constant (K_A) was calculated using Eq. (1).

$$K_A = 1 \Big/ K_D = k_a \Big/ k_d \quad (1)$$

2.4. Thermodynamic data analysis

The thermodynamic data analysis was elaborated by Ross et al. [16]. The measurement of k_a and k_d values at various temperatures enables the calculation of the thermodynamic parameters associated with complex formation and dissociation. The change in Gibbs free energy (ΔG) indicates whether a reaction is spontaneous (negative ΔG) under the specified conditions or not. It is related to the equilibrium association constant (K_A) according to the following equation:

$$\Delta G = -RT\ln K_A \quad (2)$$

where $R = 8.314$ J K^{-1} mol^{-1} is the real gas constant. Additional information upon complex formation can be obtained through the evaluation of independent thermodynamic parameters such as the enthalpy or change of heat content (ΔH), and the constant pressure heat capacity change (ΔC_p). ΔH contributes to ΔG through the following relationship:

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

whereas ΔS is the entropy change. ΔH can be measured directly by calorimetry. The dependent parameter ΔS can be calculated if both values of ΔG and ΔH are known. If the enthalpy change (ΔH) does not vary with the temperature, the Van't Hoff plot of ln K_A versus $1/T$ results in a straight line of slope $-\Delta H/R$.

$$\ln K_A = -\Delta H/RT + \Delta S/R \quad (4)$$

However, very often ΔH and ΔS show significant

Table 1
Experimental conditions for the kinetic analysis

Interactant	Temperature (°C)	Buffer supplement	Flow-rate association (μl/min)	Analyte contact time (s)	Dissociation time (s)	Regeneration
GFP/M1	5–35 (each 5 °C)	5 m*M* $CaCl_2$	30	180	600	100 m*M* EDTA-HBS, 0.5% SDS
GFP/M1	5–35 (each 5 °C)	10 m*M* EDTA	30	180	600	100 m*M* EDTA-HBS, 0.5% SDS

temperature variations [27]. By introducing a temperature independent heat capacity change ΔC_p and applying the integrated form of the Van't Hoff plot [28], temperature variation of enthalpy can be taken into account.

$$\ln K = \ln K_0 + \frac{\Delta H_0 - T_0 \Delta C_p}{R}\left(\frac{1_0}{T_0} - \frac{1}{T}\right) + \frac{\Delta C_p}{R}\ln\left(\frac{T}{T_0}\right) \quad (5)$$

In Eq. (5) T_0 is an arbitrarily selected temperature, averaged from the analyzed temperature interval in Biacore experiments, K_0 is the corresponding equilibrium constant and ΔH is the Van't Hoff enthalpy at that temperature. The presumption of ΔC_p being temperature-independent is receivable over the temperature interval involved.

2.5. Approximation of the integrated Van't Hoff plot

At sufficient small temperature intervals the second term in Eq. (5) could be expanded in a Tayler series of powers $\theta = T/T_0 - 1$

$$\ln K = A + B\theta + C\theta^2 \quad (6)$$

where $A = \ln K_0$, $B = \Delta H_0/RT_0$, and $C = -\Delta H_0/RT_0$.

Insertion of $T/T_0 - 1$ for θ into Eq. (6) and rearrangement results in:

$$\Delta H_0 = \frac{\ln KRT - \ln K_0 RT_0}{(\theta - \theta^2)} \quad (7)$$

Rewriting of Eq. (5) yields in ΔC_p

$$\Delta C_p = \frac{\ln KR - \ln K_0 R - \Delta H_0\left(\frac{1}{T_0} - \frac{1}{T}\right)}{-T_0\left(\frac{1}{T_0} - \frac{1}{T}\right) + \ln\left(\frac{T}{T_0}\right)} \quad (8)$$

ΔH as a function of T was calculated by incorporating estimated parameters ΔH_0 and ΔC_p into Eq. (9):

$$\Delta H(T) = \Delta H_0 + \Delta C_p(T - T_0) \quad (9)$$

The ln K values were plotted against θ and approximated by Eq. (6) using a least square fitting algorithm.

3. Results

3.1. Interaction of anti-FLAG antibody M1 with the FLAG peptide

The binding of monoclonal antibody anti-FLAG M1 to the FLAG peptide at various temperatures as well as in the presence and absence of Ca^{2+} ions was studied using the Biacore 2000 system. Purified FLAG-GFP fusion protein was injected into the chip at various temperatures as described in the Experimental section. The obtained sensorgrams in the presence and absence of calcium are shown in Fig. 1. Similar response curves were obtained although gained response units in the presence of calcium were higher, especially in the low temperature range. k_a and k_d values attained at different temperatures under binding and non-binding conditions are shown in Fig. 2. The binding behavior at various temperatures did not show a significant difference in the presence and absence of Ca^{2+} ions. This might be due to the bivalent nature of the whole antibody M1 used in affinity chromatography and BIACORE experiments. This was also confirmed by ELISA experiments with the whole antibody M1 and its F_{ab} fragments [13]. The association equilibrium constants were calculated according to Eq. (1). The evaluated equilibrium constants at various temperatures in the presence and absence of calcium are shown in Fig. 3.

3.2. Estimation of ΔH as a function of T

ln K_A values versus $1/T$ showed a curvature suggesting a temperature dependence of ΔH. The values of the thermodynamic parameters, ln K, ΔH (at $T = 25\,^{\circ}C$) and ΔC_p obtained from the quadratic approximation of the Van't Hoff equation (Eqs [5–9].) in the presence and absence of calcium are reported in Table 2. Although a noticeable variability of the enthalpies was obvious at any temperature, a distinct tendency was in evidence. ΔH was increased steadily with temperature. As can be seen in Fig. 4. ΔG varies only little with temperature, indicating that the change of $T\Delta S$ with temperature was compensated by the change in enthalpy. This effect can be seen under binding (5 m*M* Ca^{2+}) as well under non-binding (10 m*M* EDTA) conditions.

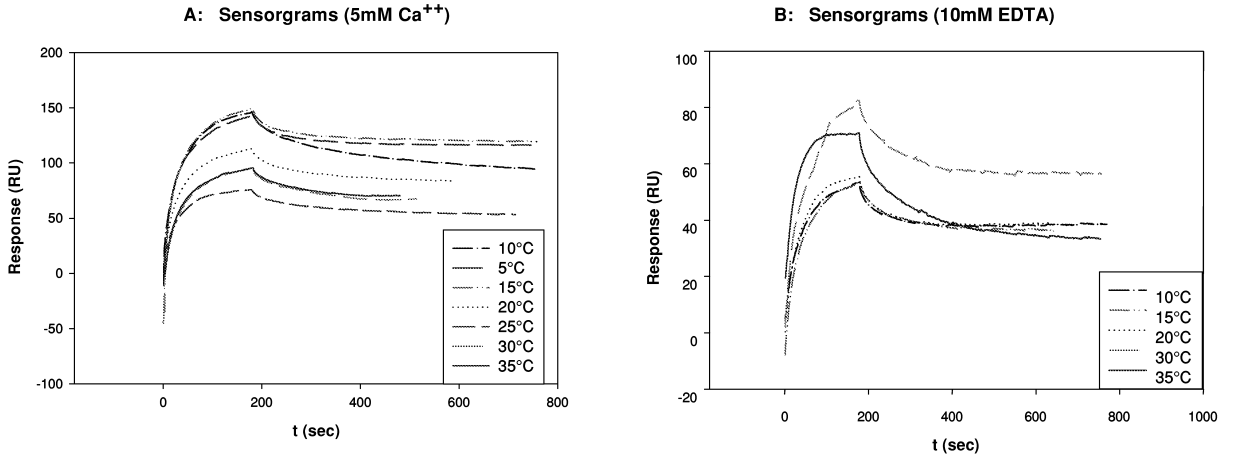

Fig. 1. Sensorgrams for all temperatures (5, 10, 15, 20, 25, 30 and 35 °C) of the interaction of anti-FLAG antibody M1 with the FLAG tagged fusion protein. (A) Data obtained in the presence of 5 m*M* calcium; (B) Data obtained in the presence of 10 m*M* EDTA (data for 5 and 25 °C not shown).

4. Discussion

The fact that kinetic measurements with the Biacore system can be performed at various temperatures provides additional insights into protein–protein interaction from a thermodynamical point of view. Besides kinetic information, thermodynamic parameters provide supplementary information on the energetic contribution to biomolecular interactions leading to a better understanding of complex formation and dissociation. In contrast to microcalorimetry where components such as hydration effects are also involved into the evaluation, Biacore technology only measures the interaction of two reactants at the sensor chip surface resulting in a change of refractive index [19]. Another advantageous feature of Biacore technology is its wide measurement range comprising most of the identified affinities for biomolecular interactions [25,29]. Complex formation of anti-FLAG antibody M1 and its

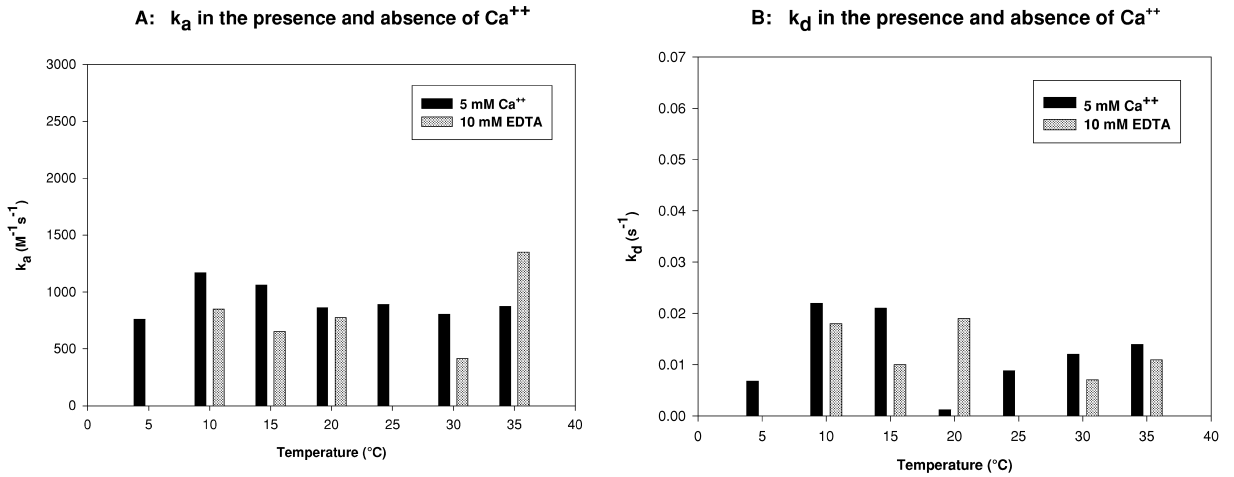

Fig. 2. Kinetic rate constants of anti-FLAG antibody M1 at various temperatures. (A) Association rate constants in the presence and absence of 5 m*M* calcium. (B) Dissociation rate constants in the presence and absence of 5 m*M* calcium.

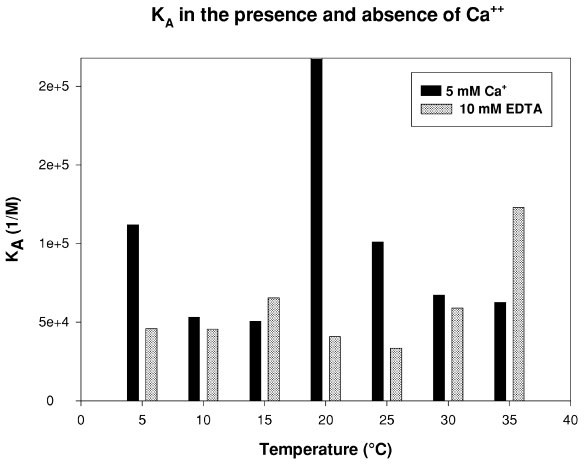

Fig. 3. Equilibrium rate constants of anti-FLAG antibody M1 at various temperatures in the presence and absence of 5 m*M* calcium.

Table 2
Thermodynamic parameters of the interaction of anti-FLAG antibody M1 directed against the FLAG tag at 25 °C using the quadratic approximation of the integrated Van't Hoff equation (Eqs [5–9].)

	ln K_A	ΔH (kJ M^{-1})	ΔC_p (kJ M^{-1} K^{-1})
5 m*M* Ca^{2+}	11.25	53.76	0.488
10 m*M* EDTA	10.41	−85.79	−0.94

corresponding counterpart, the FLAG peptide, is accomplished in a calcium-dependent manner. Dissociation can be effected by the addition of chelating agents such as EDTA. Contradictory to this theoretical background, elution in immunoaffinity chromatography is characterized by intense tailing. Subsequently, ELISA experiments with whole anti-FLAG antibody M1 even showed a calcium-independent binding behavior. From kinetic data obtained from previous Biacore experiments, elution should not be possible [20]. Based on these findings kinetic measurements were performed at seven temperatures in the range from 5 to 35 °C. The response curves did show similar devolution although there was a decrease in the obtained response units at higher temperatures in the presence of calcium. The overall measured response units under non-binding conditions were also halfway in comparison with the measurements performed in the presence of calcium. The obtained rate constants did not show a big temperature dependency, either in the presence or in the absence of calcium ions. Evaluated data for equilibrium constants did not show very high affinities for the complex formation. This might be an explanation for the calcium-independent binding behavior of anti-FLAG antibody M1 to the FLAG peptide in the presence of chelating agents such as EDTA. The evaluation of the rate constants k_a and k_d

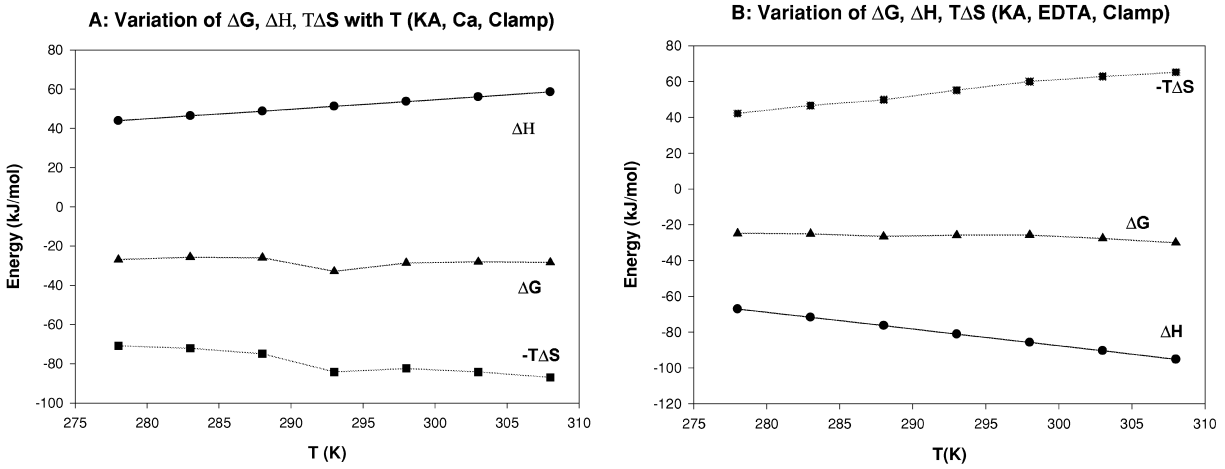

Fig. 4. Temperature variation of thermodynamic parameters ΔG, ΔH and $T\Delta S$ of the interaction of anti-FLAG antibody M1 and the FLAG tag obtained from the integrated form of the Van't Hoff equation (Eq [5].). (A) Variations in the presence of 5 m*M* calcium. (B) Variations obtained in the presence of 10 m*M* EDTA.

at various temperatures also enabled the calculation of the thermodynamic parameters such as the change in enthalpy associated with the complex formation of anti-FLAG antibody M1 and the FLAG peptide by using the Van't Hoff equation: $d \ln K_A/d(1/T) = -\Delta H/R$. The corresponding values did not show a straight line indicating a temperature dependency of ΔH. By using the integrated Van't Hoff plot and introducing a non-vanishing but temperature-independent heat capacity change ΔC_p, the temperature dependency of ΔH can be taken into account whereas assumption of a temperature-independent ΔC_p is generally valid over the limited temperature range involved in the experiment. The importance and the generality of inclusion of the temperature variation of enthalpy in the Van't Hoff equation was emphasized by Weber [30]. From thermodynamics the complex formation of anti-FLAG antibody M1 and the FLAG peptide is characterized by enthalpy entropy compensation. For binding studies in the presence of calcium both ΔH and ΔS increase with increasing temperature leading to a relatively constant ΔG. In contrast, ΔH and ΔS values obtained in the absence of calcium decrease with increasing temperature. The Gibbs free energy ΔG does not change although the contribution of enthalpy change (ΔH) and entropy change (ΔS) 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 $\Delta H - T\Delta S$. The energy changes can be assigned to mainly entropy contribution in the presence of calcium whereas the complex formation in the absence of calcium is enthalpy driven. This enthalpy–entropy compensation could serve as one explanation for the intense tailing in the elution profile in immunoaffinity chromatography. We hypothesize as follows: upon addition of the chelating agent a conformational change in the complex takes place. A transition state with less affinity compared to the equilibrium state in the presence or absence of calcium ions is formed enabling the release of the FLAG peptide from the anti-FLAG antibody M1. Further studies are required to corroborate our findings. One possibility is in assessing fluorescence shifts upon addition of calcium and finally a three-dimensional model of the complex in both states will bring an insight into the conformational change in relation to calcium removal.

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