



**Abstract:** Applied Photophysics' SX20 Stopped-Flow spectrometer may be used to measure fluorescence changes on the millisecond timescale. The technique can give important kinetic information regarding biomolecular interactions as demonstrated in this application note. Here, the kinetics of  $\text{Ca}^{2+}$  dissociation from the messenger protein Calmodulin is measured, observing changes at different temperatures with different variants of the protein. The fluorescence signal is provided by internal Tyrosine residues within the protein.

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## SX SERIES APPLICATION NOTE

# Measuring Intrinsic Tyrosine Fluorescence with Stopped-flow for $\text{Ca}^{2+}$ Kinetics of Calmodulin

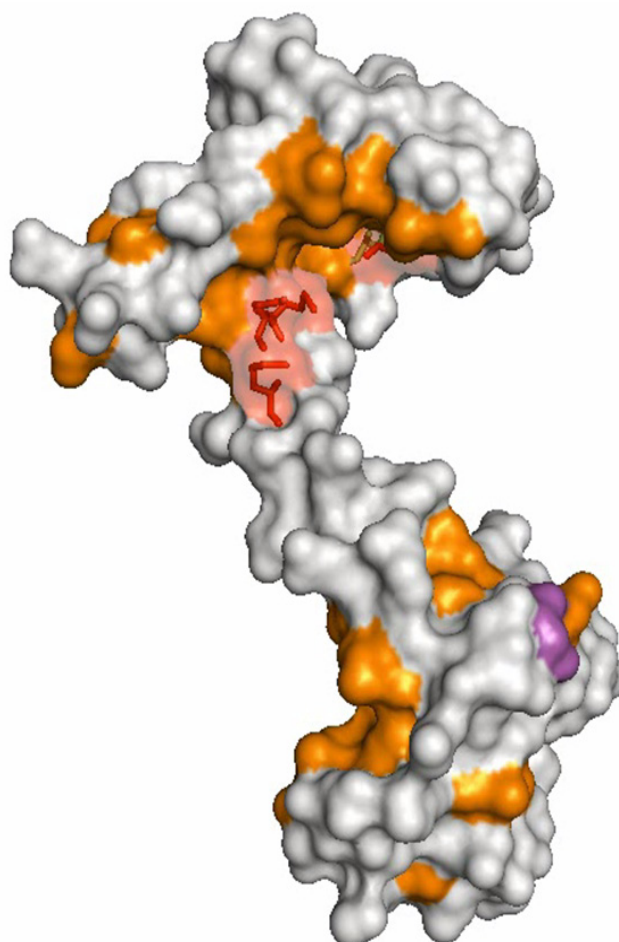


Image 1. Molecular surface of Calmodulin

## KEYWORDS

- |                                  |                      |
|----------------------------------|----------------------|
| ▶ Kinetics                       | ▶ Calmodulin(CaM)    |
| ▶ SX20 Stopped-flow spectrometer | ▶ Protein signalling |
| ▶ Fluorescence                   | ▶ Tyrosine           |
| ▶ $\text{Ca}^{2+}$ dissociation  | ▶ Spectroscopy       |

INTRODUCTION

Calmodulin, a Calcium Signalling Protein

Calmodulin(CaM) is a small amphiphilichelical protein containing 148 amino-acids (17 kDa) and is found in all mammalian cells where it functions as a Ca<sup>2+</sup> signalling protein. It is one of the most conserved proteins known in the vertebrate lineage and is a member of the calcium-binding EF-hand super family of proteins. CaM has two EF-hand motifs at each of its two domains, denoted the N-domain and the C-domain (See Figure 1). Each EF-hand is capable of binding one calcium ion, which occurs in a highly cooperative manner. The set of motifs found in the C-domain bind with an affinity of 10<sup>-6</sup>M and the N-domain set with a K<sub>d</sub> of 10<sup>-5</sup>M. The difference in affinity of the two domains is vital to the function of CaM as a signalling protein, where each domain plays a very different role (Masino L, 2000) (Tadross MR, 2008)(Vanscyoc W, 2002).

The C-domain is involved in local Ca<sup>2+</sup> sensing by interacting with various ion-channels, phosphatases, and kinases depending on the Ca<sup>2+</sup> levels in its immediate vicinity. The C-domain is often permanently bound to these interacting partners, thereby tethering the protein, thus priming CaM for its regulatory action. The N-domain has been shown to respond to global changes in cellular Ca<sup>2+</sup> levels by interacting with target proteins following oscillations in global Ca<sup>2+</sup> concentrations.

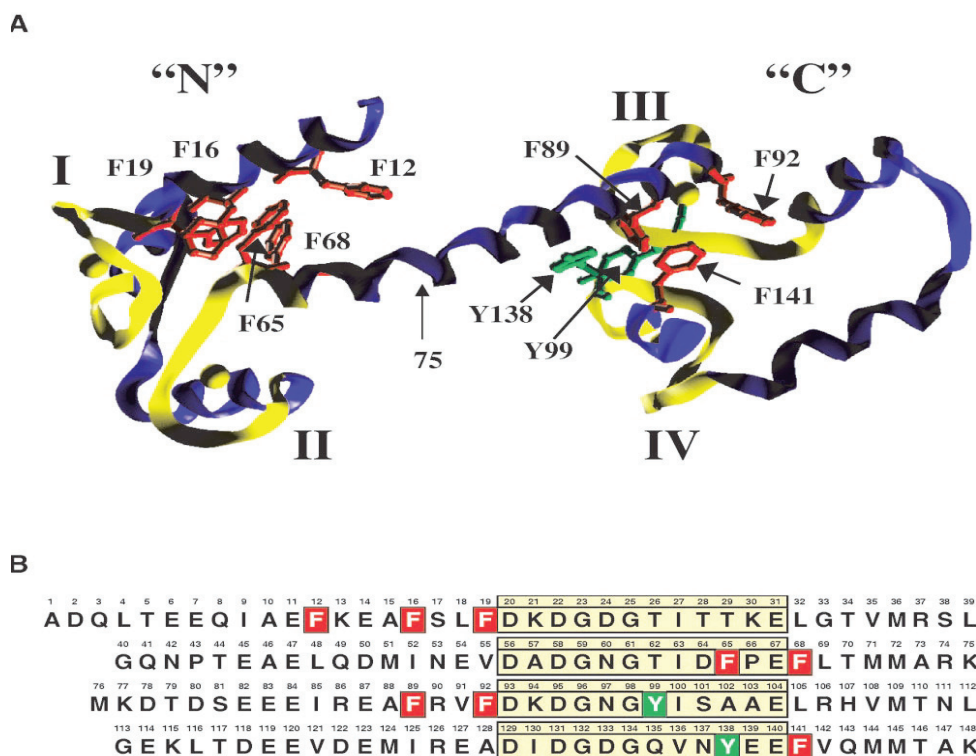


Figure 1 A) Cartoon-model of mammalian Calmodulin with the 4 EF-hands denoted I-IV and the N-domain to the left and C-domain to right. The phenylalanine residues are drawn in brown-stick form and denoted with their corresponding primary sequence number. The two Tyrosine residues found in the C-domain are coloured in blue and numbered accordingly. Calcium ions are shown as yellow spheres. B) Primary amino acid sequence of Calmodulin with phenylalanine in red, Tyrosine in green and the 4 EF-hands in yellow. Adapted from (Vanscyoc W, 2002).

This advanced dual-switch action of CaM is necessary due to the large number of proteins it interacts with, enabling the regulation of such diverse processes as memory, heart contraction, skeletal muscle contraction and egg fertilization (Masino L, 2000)(Tadross MR, 2008)(Vanscyoc W, 2002).

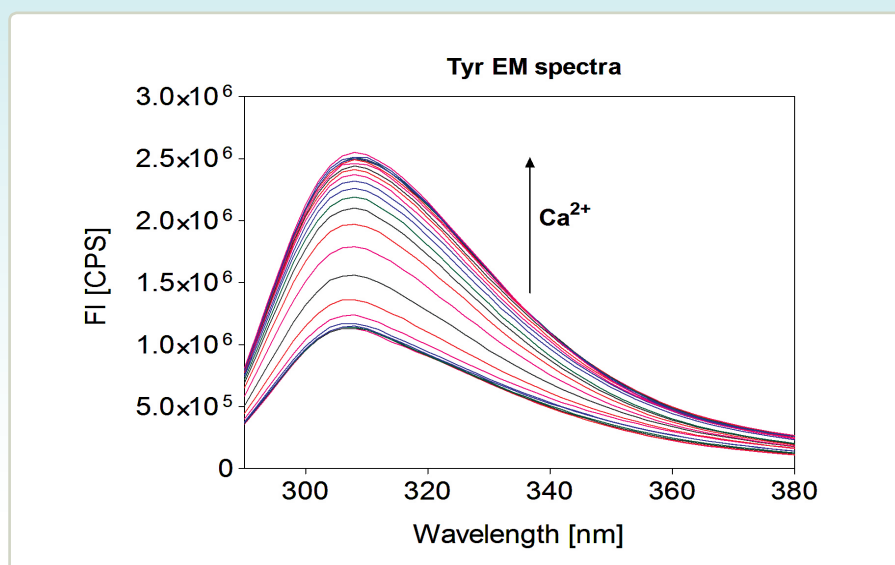
As shown by (Vanscyoc W, 2002) using steady-state and time resolved fluorescence, the fluorescent residues found in CaM are placed in a domain specific manner (see Figure 1).

The C-domain of CaM contains several fluorescent residues; there are two Tyrosine and three phenylalanine residues. There are no Tyrosine residues in the N-domain allowing the two Tyrosine residues to be utilised as reporters of changes to this domain during Ca<sup>2+</sup> binding. According to (Vanscyoc W, 2002), the phenylalanine residues in the C-domain are non-emissive due to FRET transfer to the two Tyrosine residues (the emission spectra of the phenylalanine residues overlap the absorbance of the Tyrosine residues). The N-domain contains a further five phenylalanine residues which can be used to monitor conformational changes in this domain (the experiments in this study will only consider C-domain interactions).

This approach of measuring domain specific calcium affinity of Calmodulin using intrinsic fluorescence will be performed here, except a stopped-flow approach for obtaining kinetic data will be used instead of steady-state methods. Three different proteins will be analysed, human wildtype (WT) Calmodulin, and two variants named Mut1 (Mutation located in the C-domain) and Mut2 (Mutation located in the N-domain).

### Measuring Intrinsic Protein Fluorescence

As Calmodulin contains no tryptophan residues, the Tyrosine residue signal can be easily measured on most fluorometers for both steady-state and kinetic measurements (Vanscyoc W, 2002) (Shalitin, 1981). This is due to the high intensity of the Tyrosine emission when excited in the 270-280nm range (see Figure 2). Added to this, the signal change upon calcium binding is more than a 50% increase at full saturation, giving a large signal span for kinetic measurements.



**Figure 2.** Fluorescence steady-state measurements of Tyrosine (ext 277nm) during a titration of CaM-WT with increasing Ca<sup>2+</sup> concentration resulting in a >50% increase of the Tyrosine signal at full saturation (increasing Ca<sup>2+</sup> in the direction of the arrow). Data was recorded on a Horiba Scientific FluoroMax 4P and 15μM of CaM-WT.

As Calmodulin's binding of calcium is very rapid ( $k_{on} \approx 10^8 \text{ s}^{-1}$ ), it cannot be measured using stopped-flow techniques and will therefore not be attempted here. However, as the affinity ( $K_d$ ) of each domain can be measured using steady-state fluorescence, only one of the kinetic parameters need to be determined (either  $k_{on}$  or  $k_{off}$ ) to calculate the other using the  $K_d$  value. The off-loading of Ca<sup>2+</sup> from Calmodulin is much slower, taking place in a timeframe of seconds for the C-domain, and milliseconds for the N-domain, lending itself well to stopped-flow measurements with the SX20 instrument.

The  $k_{off}$  kinetics of Calmodulin have been determined previously (Martin, 1984), (Shalitin, 1981) and (Martin SR, 1985) for both domains using extrinsic fluorophores such as Quin-2 (fluorescent Ca<sup>2+</sup> chelator) and ANS (binds hydrophobic regions) (Table 1).

Temperature (°C)	CaM [μM]	Ca <sup>2+</sup> [μM]	Quin-2 [μM]	"N" $k_{fast}$ [s <sup>-1</sup> ]	"C" $k_{slow}$ [s <sup>-1</sup> ]
11	12.5	100	200	293±92	2.1±0.4
19	12.5	50	200	550±175	5.3±0.9
28	12.5	50	200	-	9.1±1.5

**Table 1.** CaM-WT Ca<sup>2+</sup> release kinetics measured using stopped-flow with Quin-2 as extrinsic fluorophore. (Martin, 1984)

The two main drawbacks of these approaches are firstly the possible disconnect between the rate of structural changes and ligand release. Secondly, the protein may not interact with a ligand capable of generating a secondary signal. The use of intrinsic fluorescence both mitigates and complements these problems, as the rate obtained using the intrinsic fluorescence approach is equal to the change in structure during loading/off-loading of the ligand. This enables the comparison of the rate of structural change to the rate of ligand release/binding.

## MATERIALS & METHODS

### SX20 Stopped-Flow Spectrometer Setup

The SX20 instrument used for these experiments was equipped with a 20 $\mu$ L optical cell (10mm and 2mm pathlength) and an SX/SQ Sequential mixer sample handling unit. A circulating water-bath with external manual temperature control was used to control the temperature of the reactants. The instrument was equipped with a 150W xenon arc (ozone-free) lamp and the standard excitation monochromator (Czerny-Turner). An R6095 (300-650nm) PMT (Hamamatsu) was mounted on the 10mm viewport with a WG320 Schott optical filter that has a 50% cut-off value at 320nm. An SX/SM Monochromator was connected with a fibre optic cable to the 2mm viewport and had an R6095 (300-650nm) PMT detector (Hamamatsu) mounted. The drive syringes were 1.5mL glass Hamilton syringes and the injection was set to 50 $\mu$ L from each syringe per drive.

### Measuring Tyrosine Fluorescence

The Calmodulin variants used for the experiments were produced using a Rosetta *E.coli* (Novagen,US) system and a modified pMal-fusion vector (New England Biolabs, US). Purification was performed in three discrete chromatographic steps starting with affinity chromatography (New England Biolabs, amylose resin), followed by cleavage of fusion protein, Ion-exchange chromatography (GE-HealthCare, Source 15Q resin) and a polishing step of Size-exclusion chromatography (Pharmacia, Sephadex 75-16/60). Purity and size was verified by SDS-PAGE and intact LC-Q-TOF Mass Spectroscopy. Correct folding was confirmed using an Applied Photophysics Ltd Chirascan™ circular dichroism spectrometer.

Tyrosine fluorescence kinetic measurements for all three CaM variants C-domains were performed using a solution of 50 $\mu$ M CaM, 20mM Hepes, 100mM KCl and 400 $\mu$ M CaCl<sub>2</sub>, pH 7.2 in syringe A. Syringe B contained 20mM Hepes, 100mM KCl, pH 7.2 with 1mM EDTA to induce disassociation of Ca<sup>2+</sup> from CaM. The two drive syringes were of equal volume giving a 1:1 mixing ratio in all measurement. Measurements were performed at 10, 15, 20, 25, 30 and 37°C with three measurements at each temperature point to reduce noise. The specific instrumental setup for each variant can be found in Table 2 below.

Spectra name	Ext. [nm]	Mono Ext SW	Time [s]	Points	Temperature (°C)	H V
CaM-WT 25 $\mu$ M Tyrosine	277	1/1x4,65nm	1.5(12.5 $\mu$ s)	400	10-37	700
CaM-Mut1 25 $\mu$ M Tyrosine	277	1/1x4,65nm	0.25(12.5 $\mu$ s)	50	10-37	700
CaM-Mut2 25 $\mu$ M Tyrosine	277	1/1x4,65nm	1.5(12.5 $\mu$ s)	400	10-37	700

**Table 2.** Overview of experimental settings for stopped-flow fluorescence measurements of Ca<sup>2+</sup> disassociation from Calmodulin wildtype and the two Mut1 and Mut2 variants induced by EDTA. SW = slit width, H V = high voltage. All Tyrosine measurements were performed with a WG320 Scott optical filter 320 longpass.

A one-phase decay function was used to fit the raw kinetic data for  $k_{\text{off}}$  values using non-linear regression. Each of the measurements in a triplicate was fitted separately to obtain standard deviations for the three runs. The obtained  $k_{\text{off}}$  values were converted to an Eyring plot, see Equation 1, and fit using linear regression to obtain activation energies.  $R^2$  values from the fit were used to assess if the temperature dependence of the rate followed the Eyring equation.

$$\ln \frac{k}{T} = \frac{-\Delta H^\ddagger}{R} \cdot \frac{1}{T} + \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R}$$

**Equation 1.** The linear Eyring equation,  $k$  is the rate,  $\Delta H^\ddagger$  is activation enthalpy,  $\Delta S^\ddagger$  is activation entropy,  $k_B$  is the Boltzmann's constant,  $h$  is Planck's constant and  $R$  is the gas constant.

## RESULTS

### Calmodulin C-domain Kinetics Measured by Tyrosine Fluorescence

The data obtained from the stopped-flow experiments are presented on Figure 3 a-c. As expected, a drop in the signal is seen upon Ca<sup>2+</sup> disassociation from CaM. The plots are then averaged from triplicate runs with the one phase decay function fitted in red. It can be seen from the timescales that the CaM-Mut1 variant shows a very different off-rate compared to CaM-WT and CaM-Mut2. If Figure 3-d and Figure 3-e are considered, it can be seen that none of the two variants have the same response to temperature as the wildtype. The last two temperature points for CaM-Mut1 are highly affected by the poor signal-noise ratio as much of the signal is lost in the dead-time of the instrument, due to fast kinetics. This is also evident from Figure 3-f where it can be seen that both CaM-WT and CaM-Mut2 follow the Eyringer plot with  $R^2$  values of 0.997 and 0.994, whereas the CaM-Mut1 data is less linear ( $R^2=0.959$ ), affected by the last two points. To compare the calculated rate constants, the data was collated as in Table 3 together with literature values.

Protein variant	$k_{\text{off}}^f$ [s <sup>-1</sup> ] 10°C	$k_{\text{off}}^f$ [s <sup>-1</sup> ] 25°C	$k_{\text{off}}^f$ [s <sup>-1</sup> ] 37°C
CaM-Wildtype	3.7 (0.06)	12.7 (0.31)	29.5 (1.3)
CaM-Mut1	41.5 (2.0)	152.1 (12.1)	260.5 (47.4)
CaM-Mut2	3.6 (0.04)	8.4 (0.23)	23.1 (0.74)
CaM-Wildtype (litt)	2.1	5.3	n/a

**Table 3-** Rate constants determined using stopped-flow and intrinsic Tyrosine fluorescence, ext. 277nm longpass 320nm filter. Buffer was 20mM Hepes + 100mM KCl, pH 7.2 with a final concentration of 25µM protein, 200µM Ca<sup>2+</sup> and 500mM EDTA. Data from the protein samples used in the phenylalanine measurements are also shown in which the same buffer was used with a protein concentration of 200µM. Literature values are at 11°C and 28°C from (Martin, 1984).

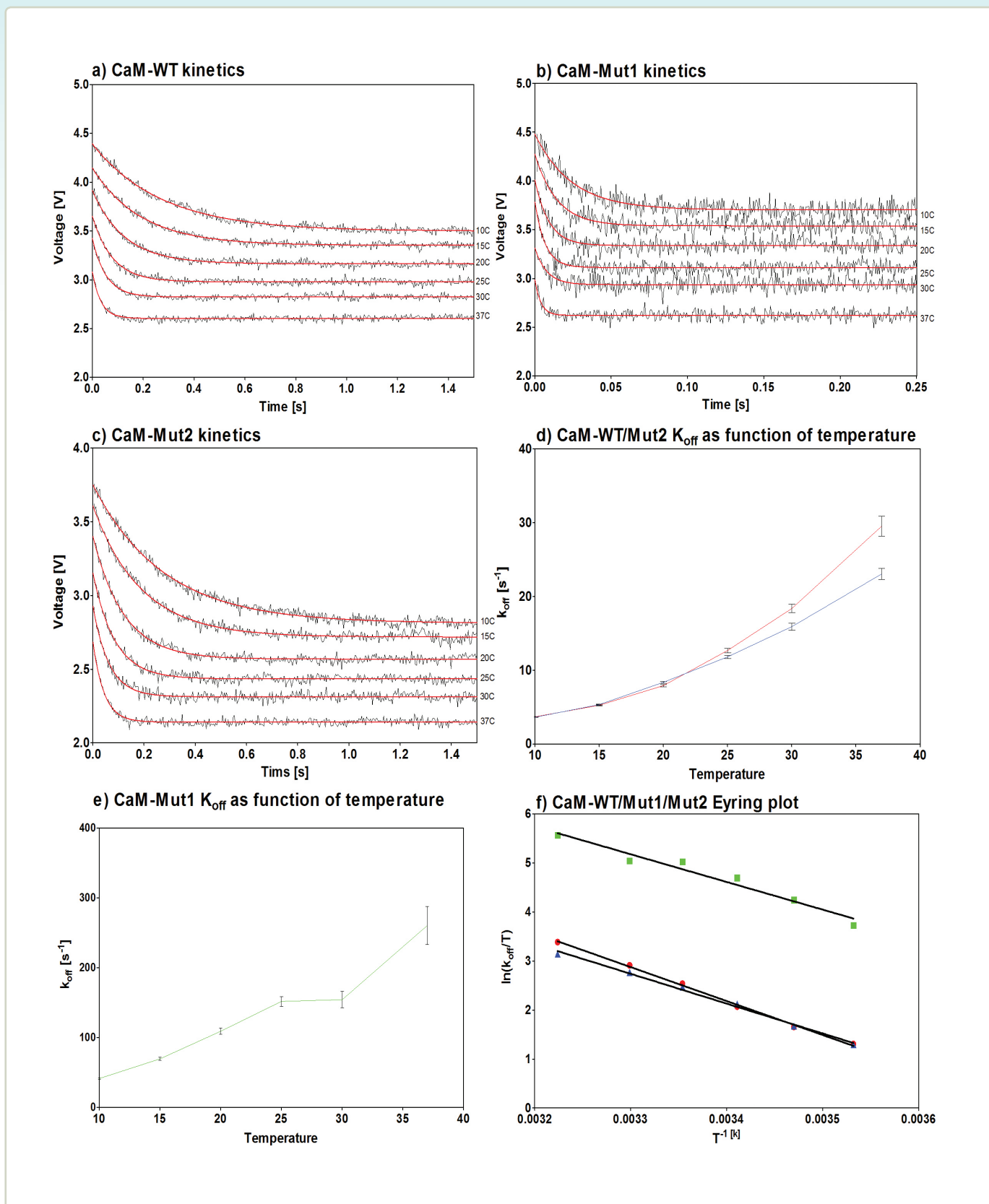
The data obtained from the fitting procedures using the Eyring equation are shown in Table 4 below. The 30°C and 37°C data points for the CaM-Mut1 were omitted from the fitting procedure due to large variations from the remaining dataset. It can be seen that the calculated values for the wildtype protein corresponds well with those reported in the literature (Martin, 1984), and that the energy barrier is lowered for Mut-1 corresponding to the higher rate constant. CaM-Mut2 has a slightly increased activation energy, in line with the lowered rate constant.

Protein type	Slope	Y-int.	$\Delta S$ (kJ/mol×T)	$\Delta G(25^\circ C)$ (kJ/mol)	$\Delta G(37^\circ C)$ (kJ/mol)
CaM-WT	-6636.1	19.1	-39.1	66.8	67.3
CaM-Mut1	-7056.3	23.0	-6.0	60.5	60.5
CaM-Mut2	-5792.8	16.1	-63.4	67.0	67.8
CaM-WT(Lit)	n/a	n/a	-30.0	67.9	67.9

**Table 4-** Slope and y-axis (y-int) obtained from fitting the data in figure 3 d-e with the Eyring equation. The activation enthalpy  $\Delta H$  and entropy  $\Delta S$  were determined using equation 1. From these values and the Gibbs-equation, Gibb's free activation energy values ( $\Delta G$ ) could be determined at 25°C and 37°C.

## DISCUSSION

Measurements of the C-domain dissociation kinetics for the three CaM variants; wildtype, Mut1 and Mut2 investigated here using intrinsic fluorescence proved successful. It was possible to determine the  $k_{off}$  rate for all three calmodulin variants at temperatures of 10, 15, 20, 25, 30 and 37°C ranging from 30 - 300s<sup>-1</sup>. The 6 rate constants determined for each protein were used in an Eyring plot, showing that the dissociation rates follow the Eyring equation. This allows lower temperature measurements to be used to predict the rate at higher temperatures. This is useful in situations where the rate at elevated temperatures is too fast to be measured, allowing the rate to be predicted instead. As an example, the last two measurements points for the CaM-Mut1 protein seem to be inaccurate due to signal:noise issues; the four low temperature points could be used to extrapolate the  $k_{off}$  values to 30°C and 37°C, the latter being more physiologically relevant.



**Figure 3.** a-c) Development of the Tyrosine fluorescent signal after EDTA induced Ca<sup>2+</sup> disassociation from CaM-WT/Mut1/Mut2. Black lines are the average of 3 runs at each temperature point. Red lines represent fitted data of  $k_{off}$  values using a one phase decay function in Graphpad Prism 5. d) Plot of CaM-WT (red) and CaM-Mut2 (blue)  $k_{off}$  values against temperature, error bars are calculated from three runs. e) Plot of CaM-Mut1  $k_{off}$  values against temperature, errorbars are calculated from three runs. f) A Eyring plot of  $\ln(k_{off}/T)$  against  $1/T$  to obtain activation energies for the release of Ca<sup>2+</sup> CaM-WT (red), CaM-Mut2 (blue) and CaM-Mut1 (green). (See table 3)



In general, the signal:noise ratio of the Tyrosine measurements can easily be improved by using higher protein concentrations. This is especially relevant for the CaM-Mut1 protein where the kinetics are much faster than the two other proteins analysed here (30s<sup>-1</sup> compared to 300s<sup>-1</sup>). The protein concentration used here was 25μM in the optical cell which could easily be increased to 50-60μM without using an excessive amount of protein. The signal could also be improved by using a more appropriate optical filter, as the filter used here cuts off a considerable amount of Tyrosine emission. A longpass 300nm cut off filter with a very sharp band-cut would be ideal as this would encompass the maxima of the Tyrosine signal at 308nm.

## CONCLUSION

The experiments were successful using the SX20 stopped-flow spectrometer. This instrument is capable of measuring rates in the 30-300s<sup>-1</sup> region using a protein concentration of 25μM. The signal:noise ratio did however become an issue above a rate of ca. 200s<sup>-1</sup> although there are simple solutions to this issue. For example, increasing the protein concentration to >25μM or performing repeat scans to average the noise out. Alternatively, the optimal measurements could be performed using a longpass 300nm cut off filter with a very sharp band-cut thereby including a larger portion of the Tyrosine emission spectra.

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