Determination of Binding Affinities and Molecular Mechanisms – case studies with nucleotide binding proteins

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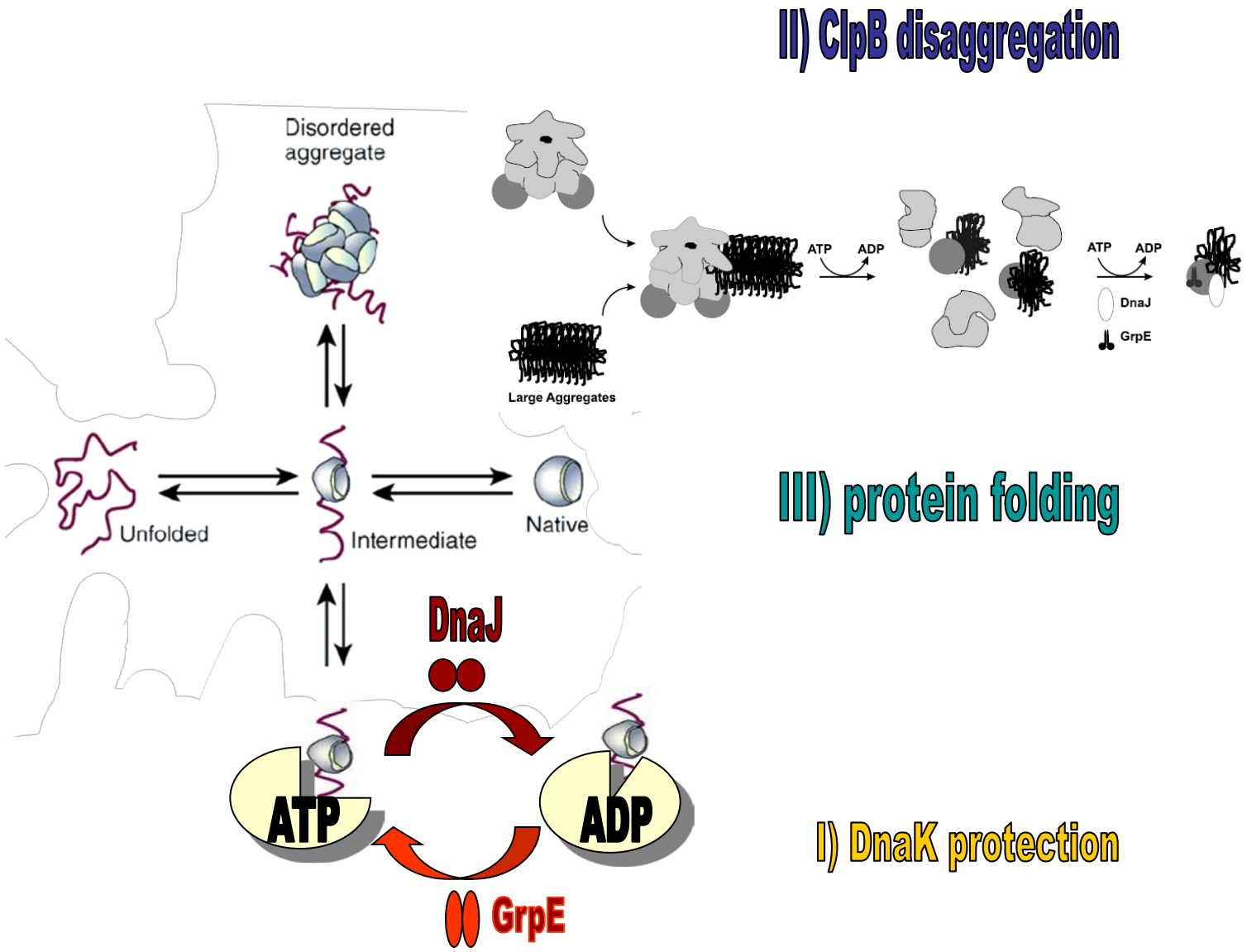
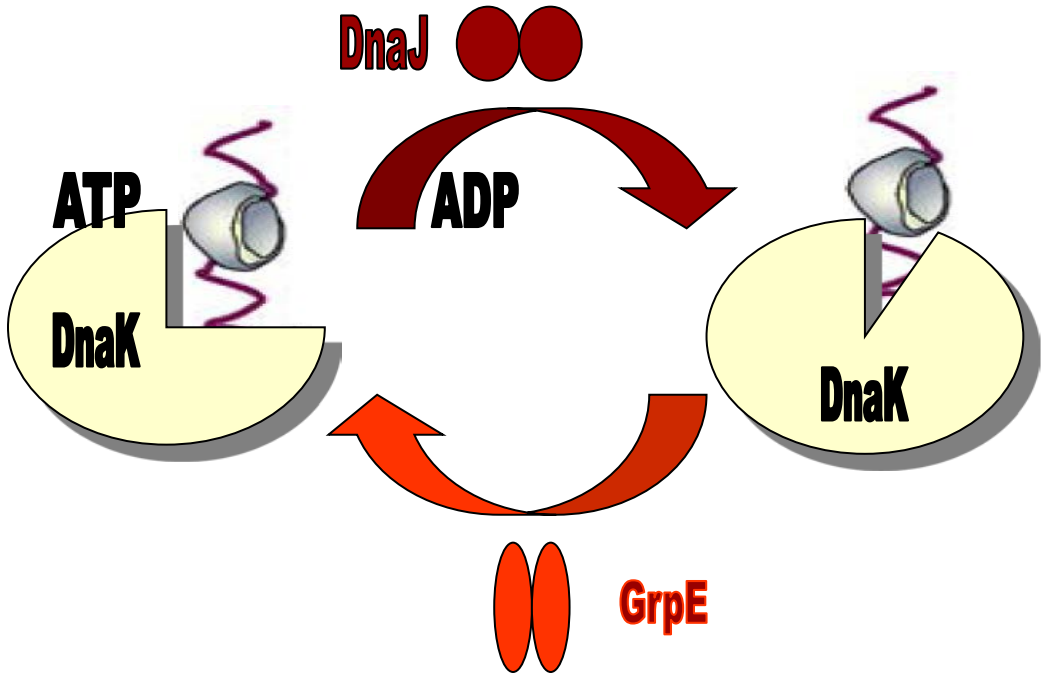
Motivation – Systems under investigation

Chaperones are ATP driven molecular machines – e.g. DnaK/Hsc70 , Hsp90 and ClpB/Hsp104



Chaperones are ATP driven molecular machines – case study DnaK/Hsc70 system



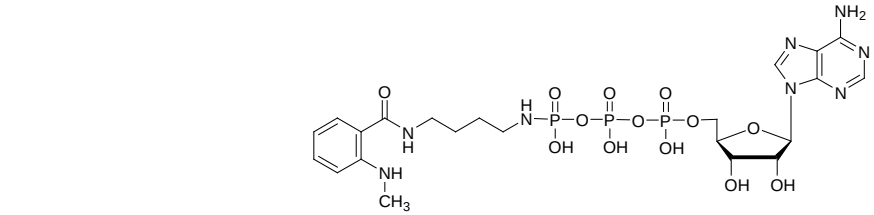
Methods/Signals to measure nucleotide binding

- Equilibrium dialysis
- Gel filtration
- NMR
- Absorption
- SPR
- Thermophoresis
- ITC
- DSC
- Fluorescence (Intensity, Anisotropy, lifetime, FRET)

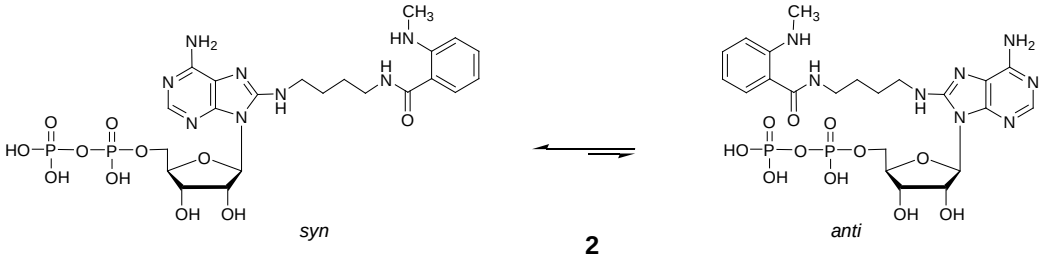
Potential origin of fluorescence signals

- Intrinsic (aa Trp, Tyr, Phe)
- Co-factors (NADH, FAD)
- Labeled Protein (e.g. fluorophore attached to Cys)
- Engineered protein (e.g. unnatural aa, GFP-tag)
- Fluorescent Nucleotide Analogs

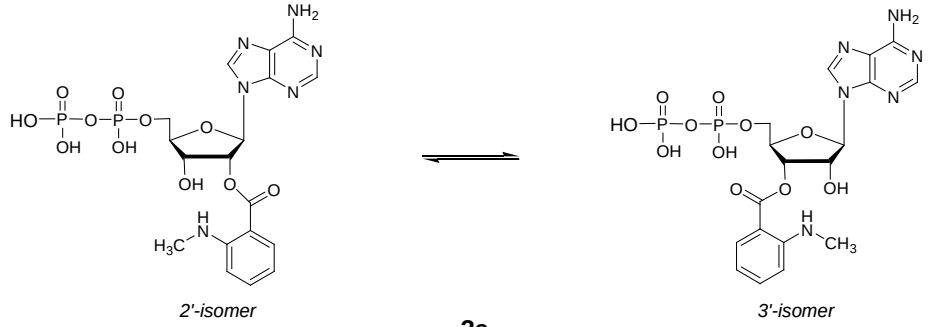
Fluorescent Nucleotides used in Chaperone Research



1



2



3a

Size of Chromophore is important, the smaller the better. ===➔

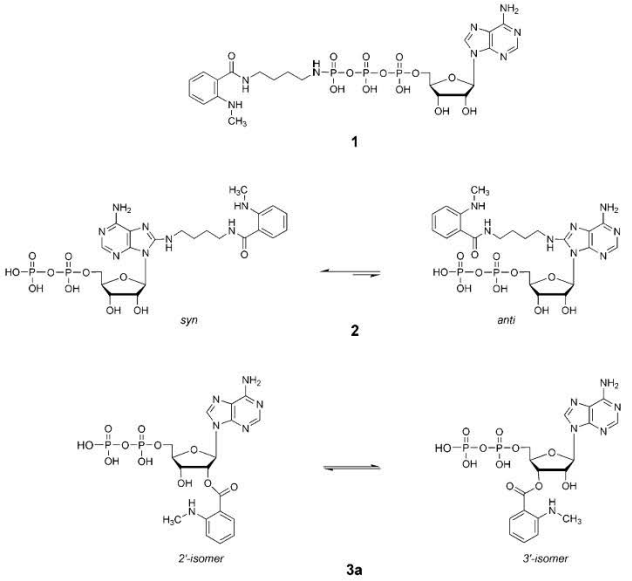
Large chromophores may disturb system to be measured

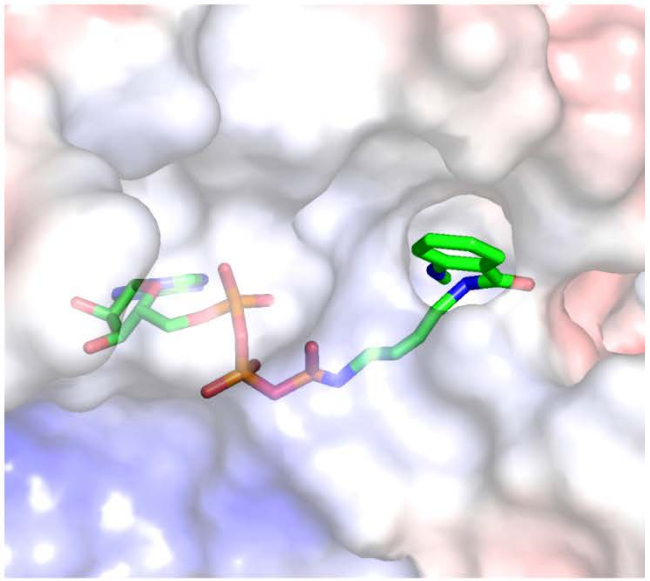
But!

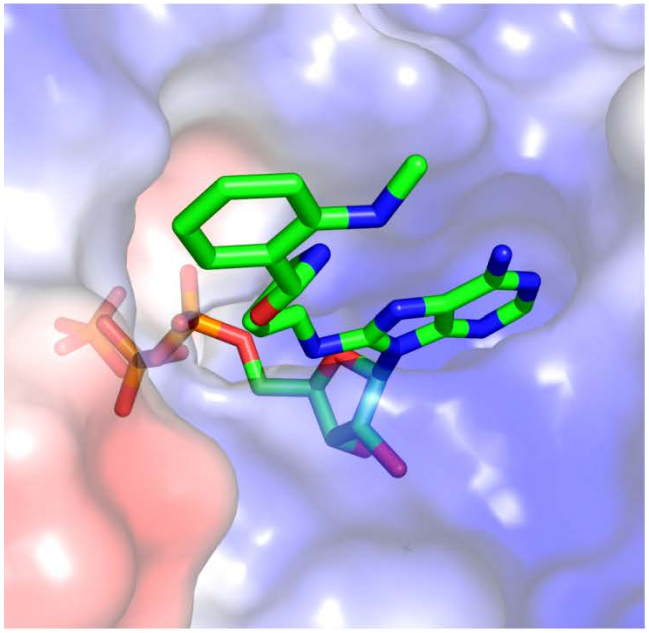
Larger chromophores have better spectroscopic properties

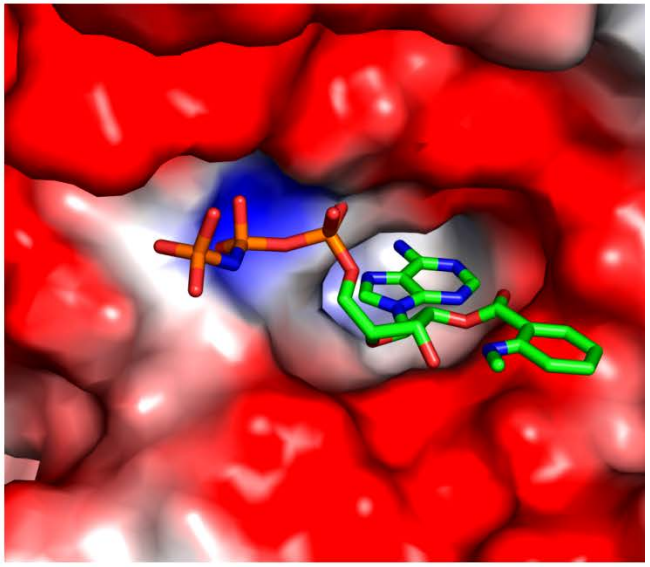
Structures of (Pγ)-MABA-ATP (**1**), (C8)-MABA-ADP (**2**) and MANT-ADP (**3a)**. For (C8)-MABA-ADP (**2**) the two conformations are shown. Unlike the other modified nucleotide analogs, the fluorescent nucleotide **2** is supposed to be mostly in the *syn*-conformation in the unbound form, while undergoing a conformational change to the *anti*-form when bound to DnaK. For MANT-ADP (**3a**) the two isomers are depicted. In solution the isomers of nucleotide **3a** are in equilibrium with 33 % of 2'-isomer and 66 % of the 3'-isomer. ===➔ **Watch out for variations caused by probes!**

Fluorescent nucleotide analogs

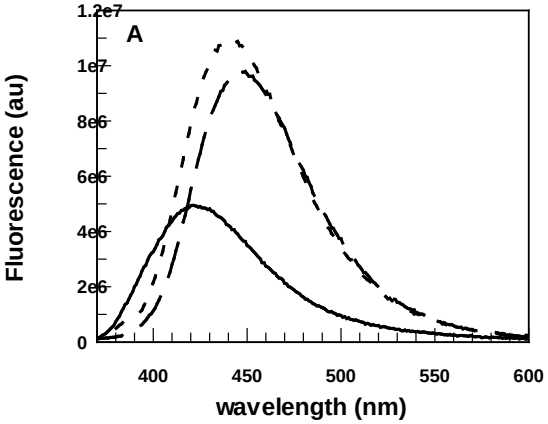


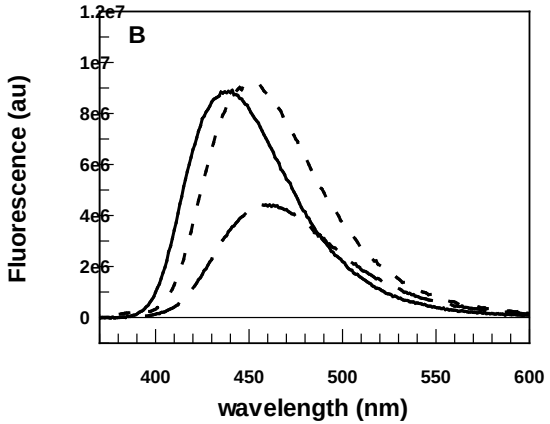


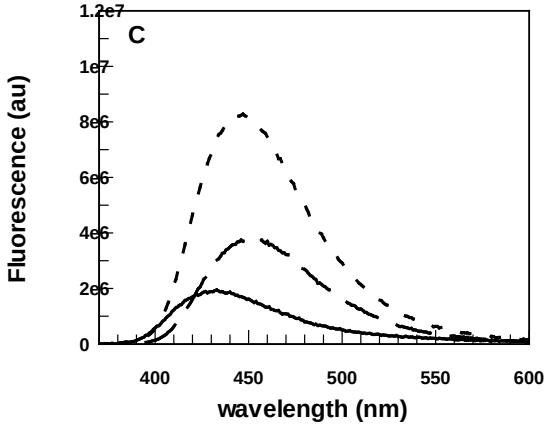


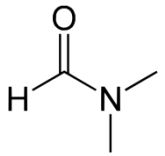


Fluorescence Spectra of Nucleotide analogs



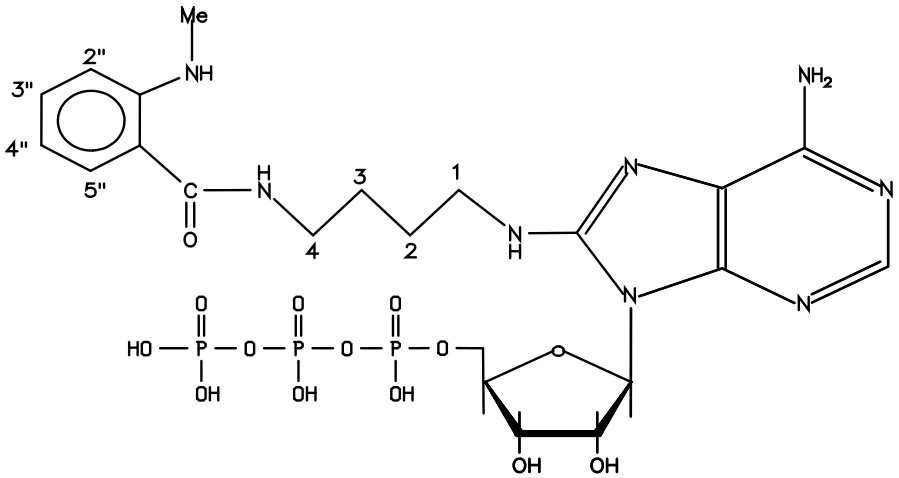






(A) Fluorescence spectra of 5 µM (Pγ)-MABA-ATP in the absence (solid in DMF, dashed in buffer) and presence of 60 µM TRAP1 (dotted). The addition of protein results in a minor increase of fluorescence while DMF reduces the fluorescence by the factor of three.
(B) Fluorescence spectra of 5 µM MANT-ADP in the absence (solid in DMF, dashed in buffer) and presence of 10 µM ClpB (dotted). The addition of protein results in a 30 % increase of fluorescence which is comparable with the emission in DMF.
(C) Fluorescence spectra of 5 µM (C8)-MABA-ADP in the absence (solid in DMF, dashed in buffer) and presence of 6 µM DnaK (dotted). The addition of protein results in a 50 % increase of fluorescence while the fluorescence signal of the nucleotide decreases by half in DMF.

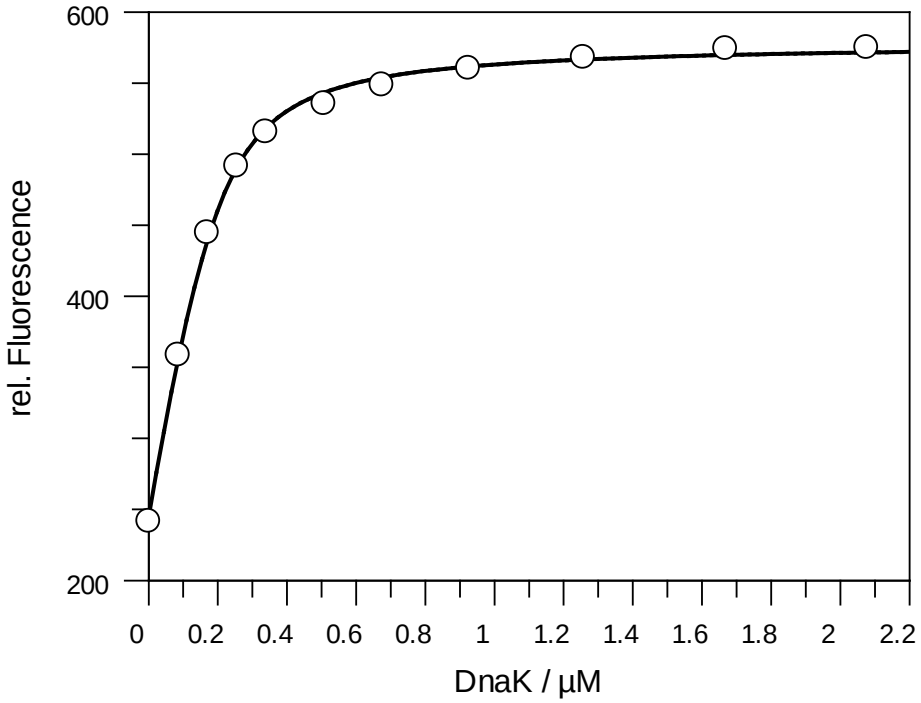
Binding of MABA-ADP to DnaK



N8-(4-N´-methylanthraniloylaminobutyl)-8-aminoadenosine 5´-diphosphate

(Theyssen et al., 1996)

Titration of MABA-ADP with DnaK (quadratic equation)



Qudratic solution, Simple w eighting

Ao = 0.1995

Reduced Chi squared = 23.83

Variable	Value	Std. Err.
Kd	0.0385	0.0037
Fo	245.2447	4.2903
Fmax	578.2120	2.9881

===➔

Titration versus premixed solutions

$$F = F_0 + \Delta F_{\max} \cdot \frac{\frac{[A_0]+[B_0]+Kd_{AB}}{2} - \sqrt{\left(\frac{[A_0]+[B_0]+Kd_{AB}}{2}\right)^2 - [A_0][B_0]}}{B_0}$$

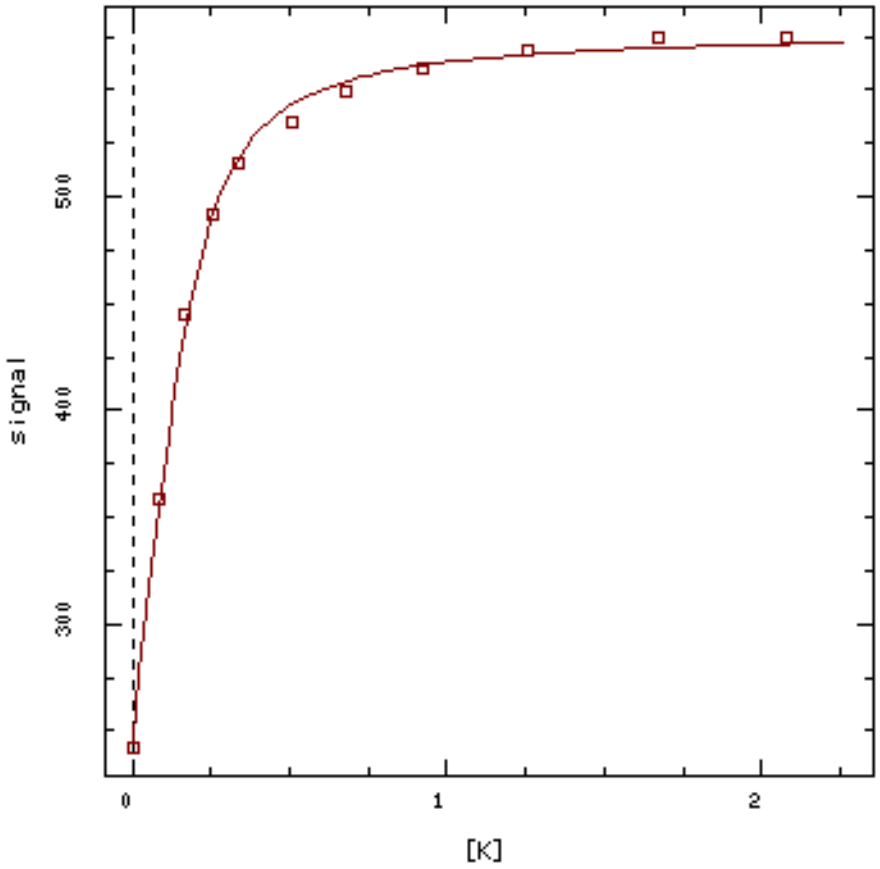
Script for simple binding

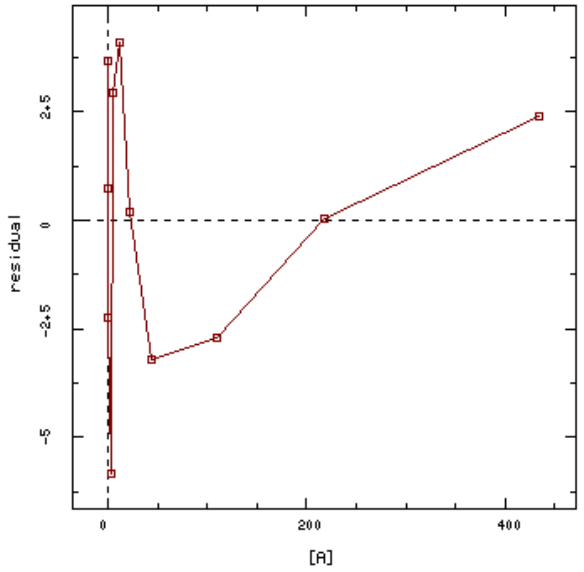
```
[task]
data  = equilibria ; equilibrium system
task  = fit ; alternative simulate
;confidence = monte-carlo; for extended error analysis
;algorithm = differential-evolution; elaborated minimum search

model = KM_binding; name of model in case several are compared

[mechanism]
 K + M <===> KM : KdKM dissoc; use Kd value
  ; binding of MABA-ADP(M) to DnaK(K)!

[constants]
   KdKM = 0.09 ??; fitted parameter

[responses] ; Signal scaling
 ;M = 1000 ?
 KM = 3000 ?

[output]
directory current\output

[data]
directory      current\data
extension      txt

variable       K ; independent variable conc. of DnaK
offset auto ? ; in case of non zero start

   file data_MABAADP_DnaK ; name of data file
   concentration  M =   0.2 ; initial conc. of MABA-ADP

[settings]
  file ./quickset.ini
[end]
```

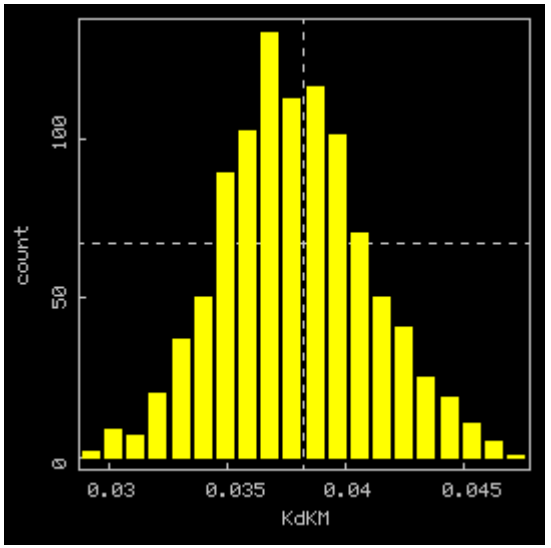
Content quickset.ini

```
{ConfidenceIntervals}
   LevelPercent            =  95 ; confidence interval
  OnlyConstants     =  y ; only "true" constants are evaluated
  MaxSteps = 50; profile-t search runs

{Output}
BlackBackground = n ; better for printing!

{MonteCarlo}
Runs = 1000 ; number of Monte-Carlo runs
```

Titration of MABA-ADP with Dank – result Dynafit analysis







title

Optimized Parameters

No.	Par#Set	Initial	Final	Std. Error	CV (%)	Note
#1	KdKM	0.09	0.0383631	0.00372551	9.71	
#2	r(M)	1000	1226.56	21.6179	1.76	
#3	r(KM)	3000	2890.8	15.0613	0.52	

Monte Carlo Method

No.	Par#Set	Mean *	Minimum	Maximum
#1	KdKM	0.0383057	0.0287728	0.0477063
#2	r(M)	1226.98	1171.7	1291.71
#3	r(KM)	2890.59	2847.94	2932.04

Variable	Value	Std. Err.
Kd	0.0385	0.0037
Fo	245.2447	4.2903
Fmax	578.2120	2.9881

Displacement of bound MABA-ADP from DnaK.MABA-ADP complex

A competitive system – cubic equation

$$A + B \underset{Kd_{AB}}{\rightleftharpoons} AB \qquad A + C \underset{Kd_{AC}}{\rightleftharpoons} AC$$

$$Kd_{AB} = \frac{[A_f][B_f]}{[AB]}; \quad Kd_{AC} = \frac{[A_f][C_f]}{[AC]}$$

$$[A_o] = [A_f] + [AB] + [AC],\ [B_o] = [B_f] + [AB],\ [Co] = [C_f] + [AC]$$

$$[AC]^3 + a_1[AC]^2 + a_2[AC] + a_3 = 0$$

$$a_o = Kd_{AB} - Kd_{AC}$$

$$a_1 = \frac{[A_o](Kd_{AC} - Kd_{AB}) + [B_o](2Kd_{AC} - Kd_{AB}) + [C_o]Kd_{AB} - Kd_{AB}^2 + Kd_{AB}Kd_{AC}}{a_o}$$

$$a_2 = \frac{[A_o][B_o](Kd_{AB} - 2Kd_{AC}) - [B_o]^2 Kd_{AC} - [B_o]Kd_{AB}([C_o] + Kd_{AC})}{a_o}$$

$$a_3 = \frac{([A_o][B_o])^2 Kd_{AC}}{a_o}$$

Cubic equations needs a search for the true solution – only one solution is valid!

$$Q \equiv \frac{a_1^{\,2} - 3a_2}{9}\,;\; R \equiv \frac{2a_1^{\,3} - 9a_1a_2 + 27a_3}{54}$$

$$Q^3 - R^2 \geq 0$$

$$\Theta = \arccos\left(\frac{R}{\sqrt{Q^3}}\right)$$

$$x_1 = -2\sqrt{Q}\cos\left(\frac{\Theta}{3}\right) - \frac{a_1}{3}$$

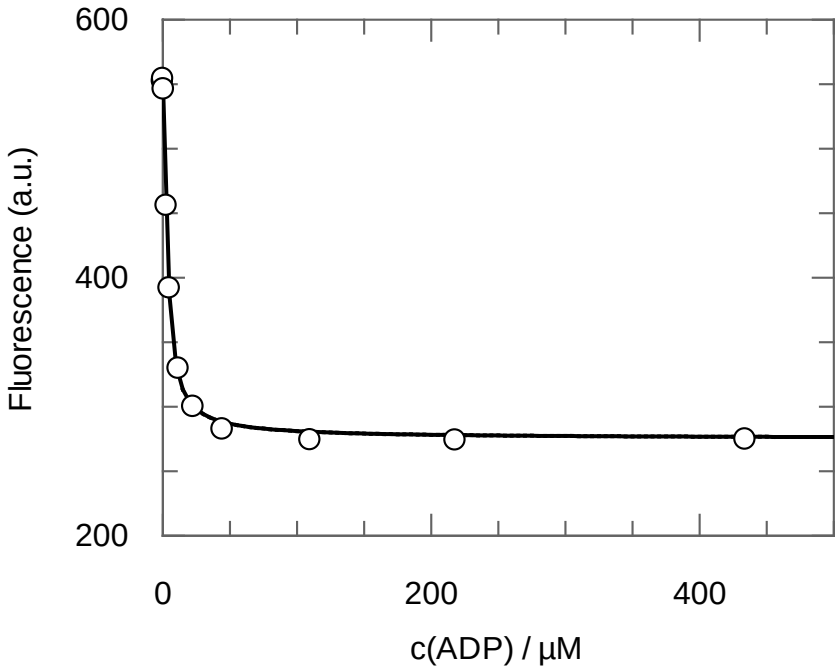
$$x_2 = -2\sqrt{Q}\cos\left(\frac{\Theta + 2\pi}{3}\right) - \frac{a_1}{3}$$

$$x_3 = -2\sqrt{Q}\cos\left(\frac{\Theta + 4\pi}{3}\right) - \frac{a_1}{3}$$

$$R^2 - Q^3 > 0$$

$$[AC] = -sign(R)\left[\sqrt[3]{\sqrt{R^2 - Q^3} + |R|} + \frac{Q}{\sqrt[3]{\sqrt{R^2 - Q^3} + |R|}}\right] - \frac{a_1}{3}$$

Displacement titration – ADP vs. MABA-ADP (DnaK) – cubic equation



Parameter	Value	Std. Error
initial fluorescence	275.2705	3.1135
Amplitude	283.5723	4.7767
Kd of competing lig.	0.0386	1.33704e-011

Initial values . ===➔
[DnaK] 2.04 µM
[MABA-ADP] 0.195
KdKM 0.0385 (from binding titration)

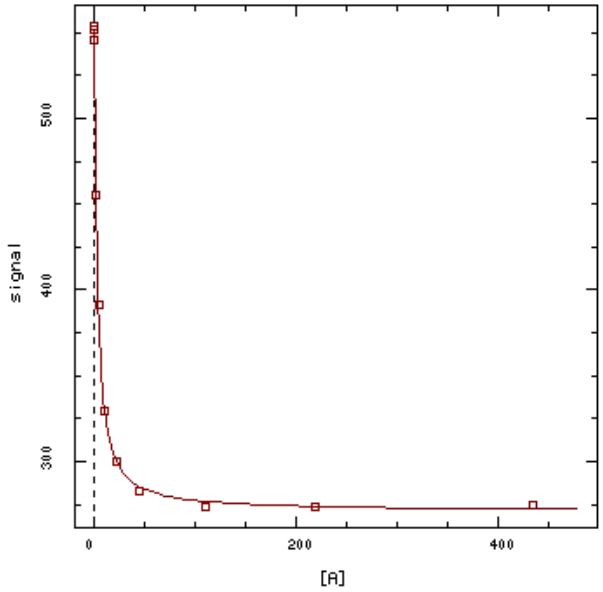
Note: Errors will be introduced through dilution effects!

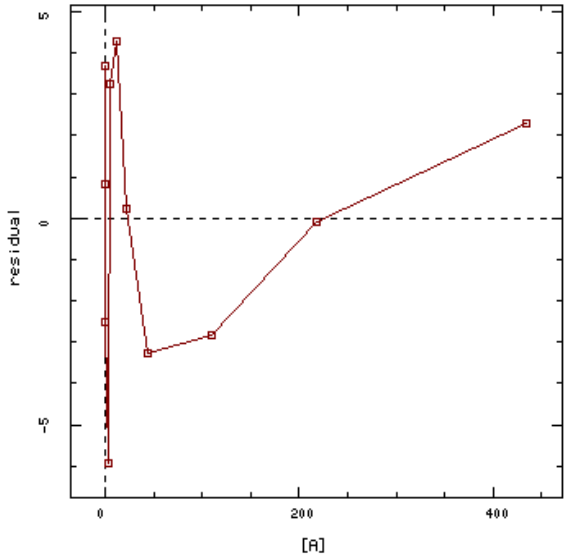
Using Dynafit for competitive displacement analysis

```
[task]
data  = equilibria
task  = fit

; confidence = monte-carlo
; algorithm = differential-evolution

model = KMA; competition of M-ADP and ADP

[mechanism]
   K + M <===> KM :  KdKM dissoc
 ; binding MABA-ADP(M) to DnaK(K)
   K + A <===> KA :  KdKA dissoc
 ; binding ADP(A) to DnaK(K)
[constants]
   KdKM = 0.1 ??;
   KdKA =0.0385 ;
[responses]
   M = 1000 ?
   KM = 3000 ?
```

```
[output]
   directory current\output
[data]
   directory      current\data
   extension      txt
   variable       A
;offset auto ?

   file MABAADP_DnaK_ADP_data
   concentration  M =   0.1915, K = 2.04

[settings]
   file ./quickset.ini
[end]
```

Displacement titration – ADP vs. MABA-ADP (DnaK) - Dynafit

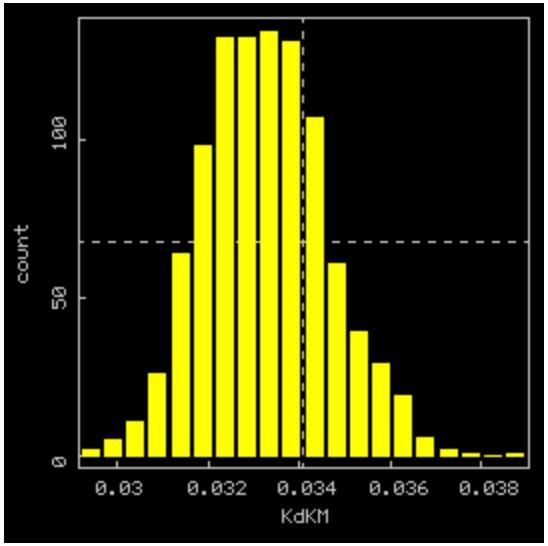




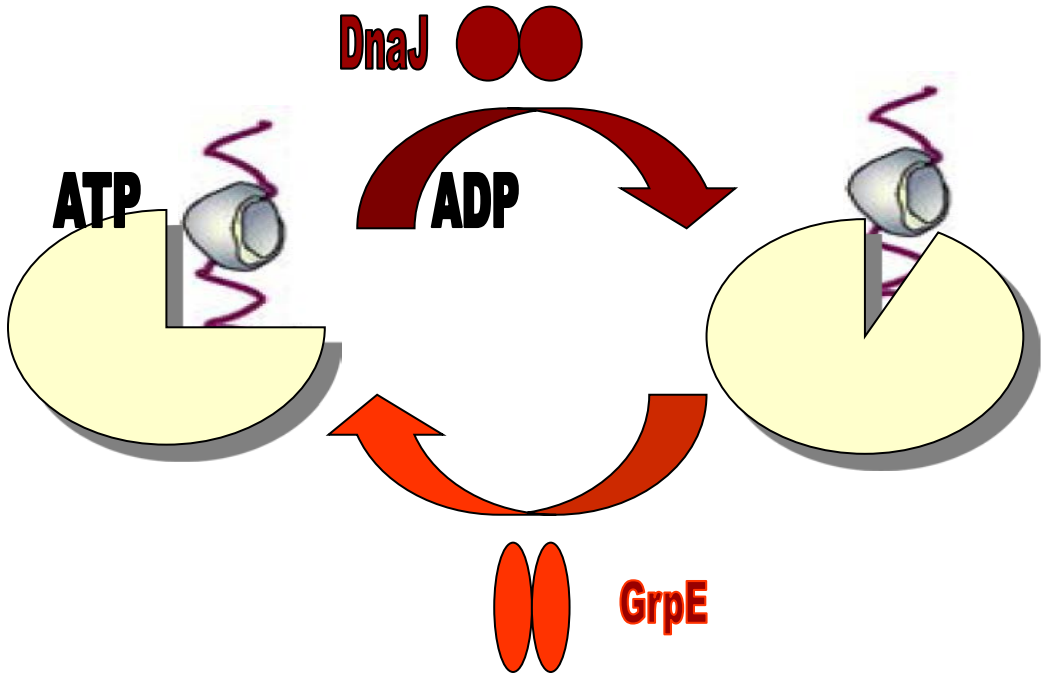
No.	Par#Set	Initial	Final	Std. Error	CV (%)
#1	KdKM	0.1	0.033458	0.00162515	4.86
#2	r(M)	1000	1414.22	9.78474	0.69
#3	r(KM)	3000	2904.03	11.8377	0.41

Monte Carlo Method Optimized Parameters

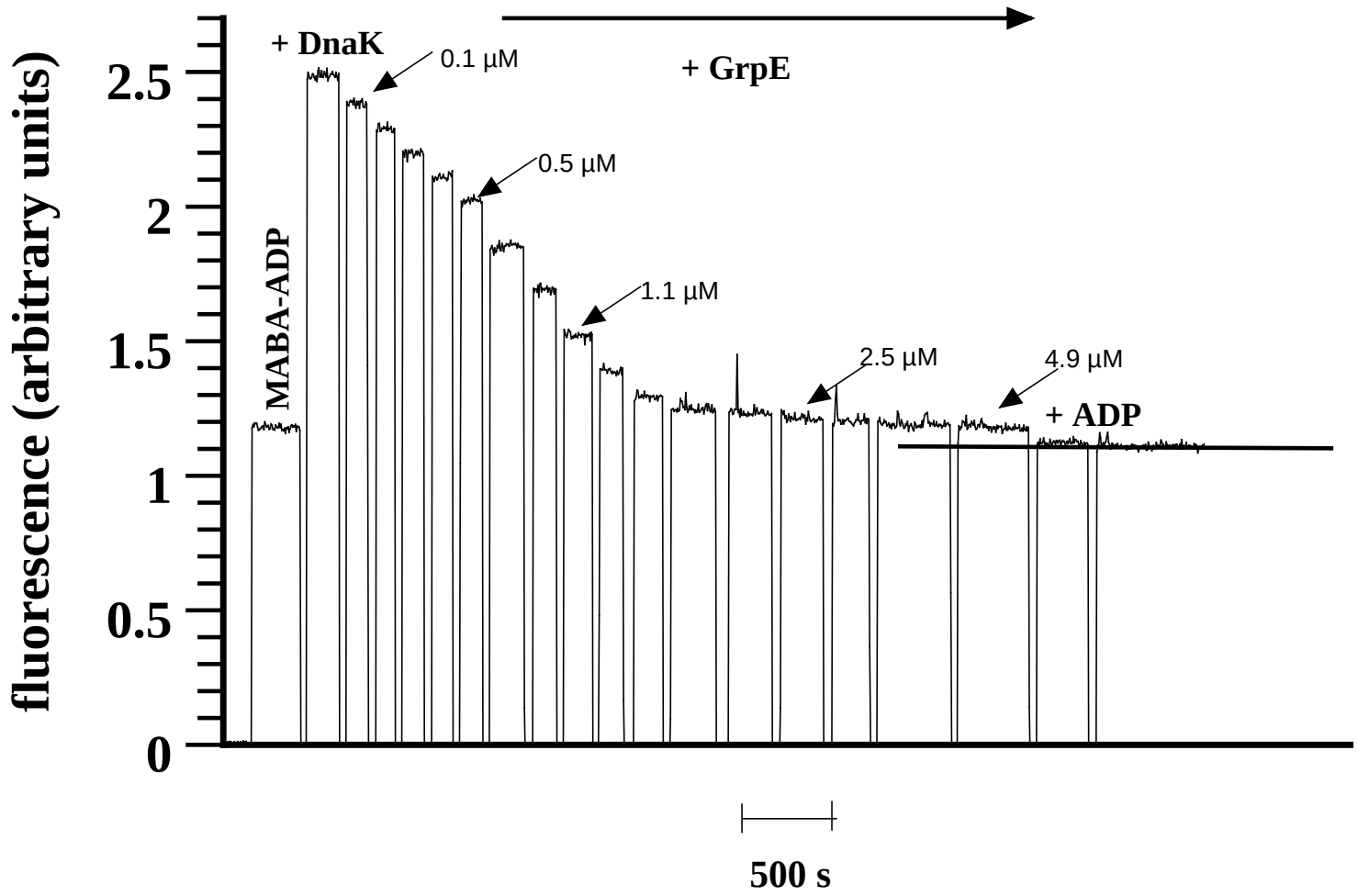
No.	Par#Set	Mean *	Minimum	Maximum
#1	KdKM	0.033445	0.0291649	0.0390008
#2	r(M)	1414.06	1386.03	1443.14
#3	r(KM)	2904.49	2871.94	2939.57



Case study DnaK/Hsc70 system – nucleotide exchange factor GrpE

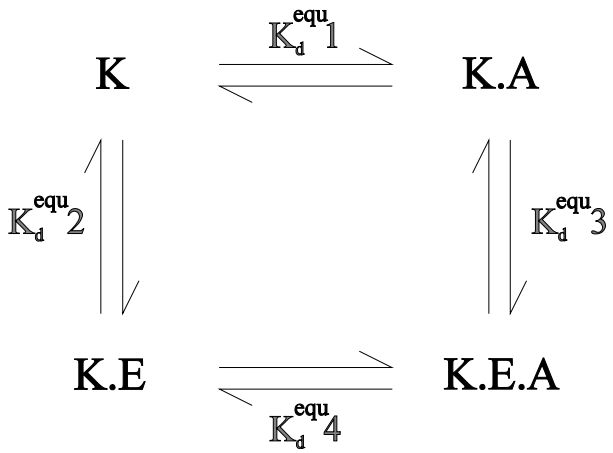


Nucleotide exchange factor GrpE – a ternary system



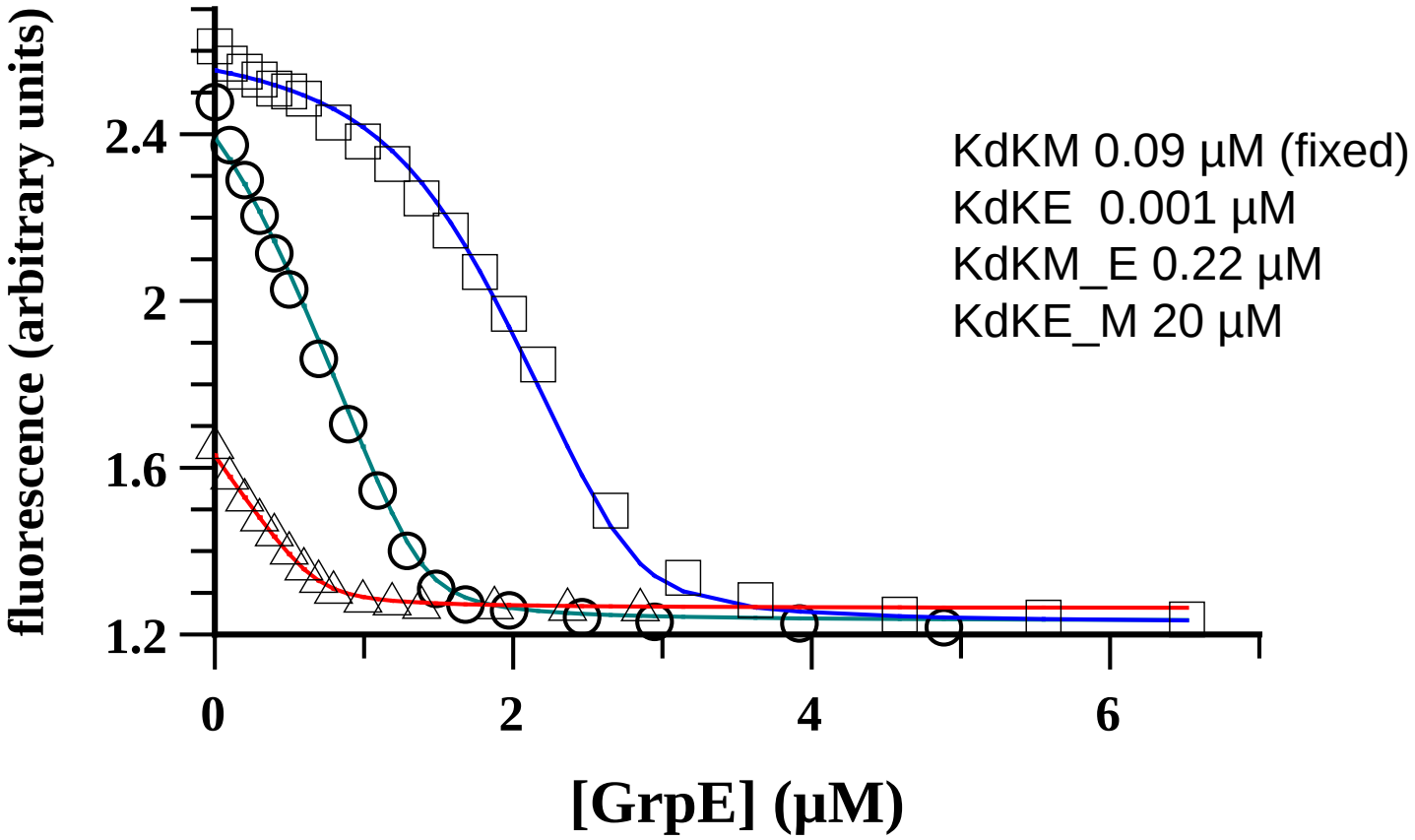
(Packschies et al., 1997)

Dna**K**-Grp**E**-MABA-**A**DP (KEM), a ternary system



$$K_d^{equ}1 = \frac{[K][A]}{[K.A]} \qquad K_d^{equ}2 = \frac{[K][E]}{[K.E]} \qquad K_d^{equ}3 = \frac{[K.A][E]}{[K.E.A]} \qquad K_d^{equ}4 = \frac{[K.E][A]}{[K.E.A]}$$

Dna**K**-Grp**E**-MABA-**A**DP (KEM), a ternary system



(□) 2 µM DnaK and 1 µM MABA-ADP; (○) 1 µM DnaK and 1 µM MABA-ADP; (△) 0.5 µM DnaK and 2 µM MABA-ADP

(Packschies et al., 1997)

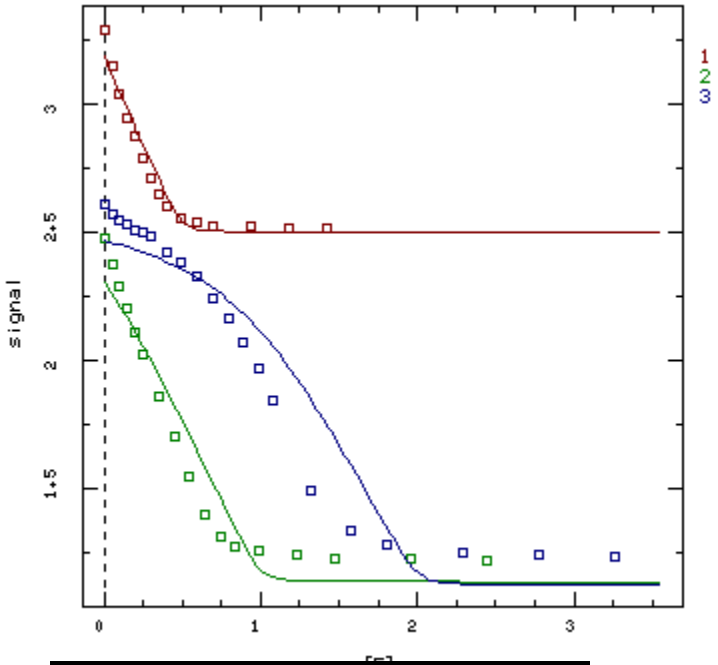
Equation for global fit of equilibrium system in scientist

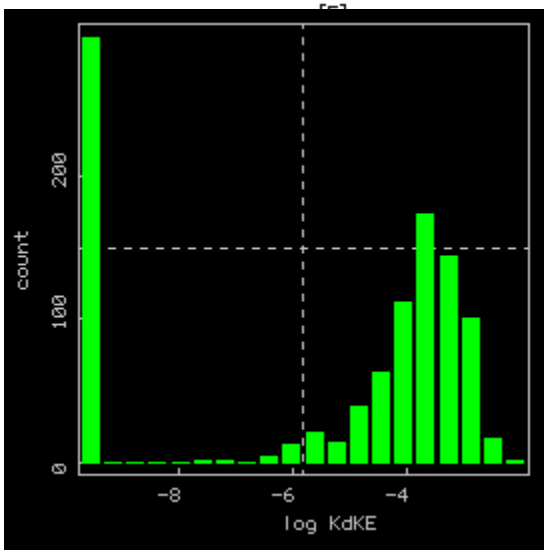
```
// GrpE : DnaK : MABA-ADP, File dnakgrpE.eqn
IndVars: C1
DepVars: A1, B1, AB1, AC1, ABC1, Cf1, F1, A2, B2, AB2,
AC2, ABC2, Cf2, F2, A3, B3, AB3, AC3, ABC3, Cf3, F3
Params: K1, K2, K3, Yb1,Yb2, Yb3,Yab1, Yab2,Yab3,
ATOT1,ATOT2,Atot3,  BTOT1,Btot2, btot3,n
AB1=(A1*B1)/K1
AC1=(A1*Cf1)/K2
ABC1=(AB1*Cf1)/K3
ABC1=(AC1*B1)*(K2/(K1*K3))
ATOT1=A1+AB1+AC1+ABC1
BTOT1=B1+AB1+ABC1
n*C1=Cf1+AC1+ABC1
F1=Yb1+(Yab1*(AB1+ABC1))/BTOT1
0<A1<ATOT1
0<B1<BTOT1
0<Cf1<n*C1
AB2=(A2*B2)/K1
AC2=(A2*Cf2)/K2
ABC2=(AB2*Cf2)/K3
ABC2=(AC2*B2)*(K2/(K1*K3))
ATOT2=A2+AB2+AC2+ABC2
BTOT2=B2+AB2+ABC2
n*C1=Cf2+AC2+ABC2
F2=Yb2+(Yab2*(AB2+ABC2))/BTOT2
0<A2<ATOT2
0<B2<BTOT2
0<Cf2<n*C1
AB3=(A3*B3)/K1
AC3=(A3*Cf3)/K2
ABC3=(AB3*Cf3)/K3
ABC3=(AC3*B3)*(K2/(K1*K3))
ATOT3=A3+AB3+AC3+ABC3
BTOT3=B3+AB3+ABC3
n*C1=Cf3+AC3+ABC3
F3=Yb3+(Yab3*(AB3+ABC3))/BTOT3
0<A3<ATOT3
0<B3<BTOT3
0<Cf3<n*C1
//Parameter values
K1=0.09
K2=0.002
K3=1.5
Yb1=1.154
Yb2=1.076
Yb3=1.224
```

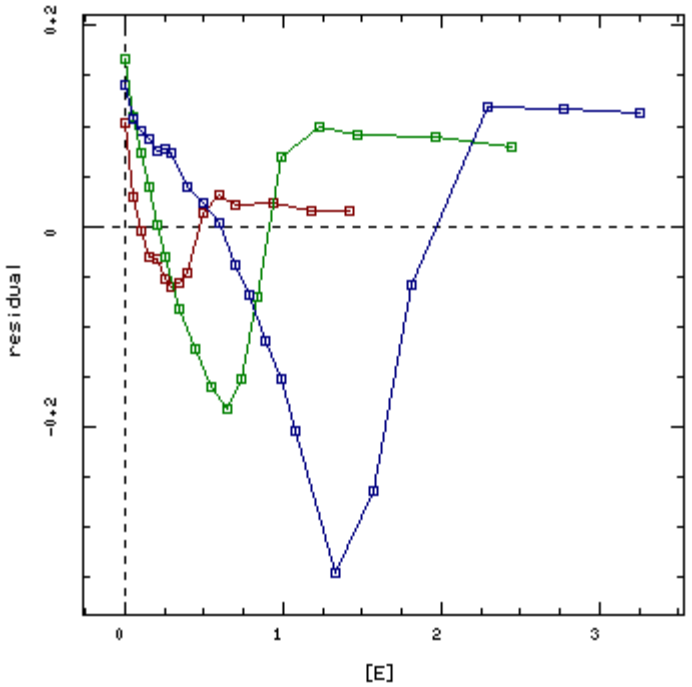
Ternary system in Dynafit

```
[task]

data  = equilibrium
task  = fit
confidence = monte-carlo

model = KEAGlobalEqu

[mechanism]

   K + M <===> KM      :  KdKM   dissoc
   K + E <===> KE      :  KdKE   dissoc
   KE + M <===> KEM      :  KdKE_M   dissoc

[constants]

   KdKM = 0.09
   KdKE = 0.001 ? ;(0.001..0.01)
   KdKE_M = 20 ? ;(1..1000)

[responses]
;  M = 1.0 ?, KM = 2.0 ?

[equilibria]

[data]
directory      current\data
extension      txt

variable       E
; offset auto ?


   file KM05_2
   conc  K = 0.5, M = 2.0
   resp M = 1.0 ?, KM = 2.0 ?, KEM = 1.0 * KM
;offset auto ?


   file KM1_1
conc K =  1.0, M = 1.0
   resp M = 1.0 ?, KM = 2.0 ?, KEM = 1.0 * KM
;offset auto ?


   file KM2_1
 conc K =  2.0, M = 1.0
   resp M = 1.0 ?, KM = 2.0 ?, KEM = 1.0 * KM
;offset auto ?


[output]
   directory current\output
[settings]
 ;  file ./quickset.ini

[end]
```

Fit ternary system dynafit







Parameters of fit ternary system dynafit

No.	Par#Set	Initial	Final	Std. Error	CV (%)	Note
#1	KdKE	0.001	0.000185668	0.000894791	481.93	
#2	KdKE_M	20	2.19451	2.56378	116.83	
#3	r(M)#1	1	0.940233	0.213096	22.66	
#4	r(KM)#1	2	3.71762	0.701888	18.88	
#5	r(M)#2	1	0.525555	0.562562	107.04	
#6	r(KM)#2	2	2.9315	0.235754	8.04	
#7	r(M)#3	1	1e-006	1.10738	110738059.25	MIN
#8	r(KM)#3	2	2.67667	0.133496	4.99	