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CARIPARO Foundation (Starting Grant 2015-2019 Carbonera/Bortolus)

Metodologie che lo studente può imparare

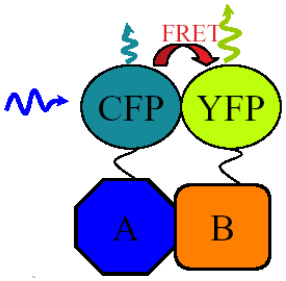
- **Metodologie di biologia molecolare** (PCR, clonaggi, mutagenesi)

- **Metodologie biochimiche** per la **purificazione** di proteine ricombinanti espresse in diversi sistemi eterologhi (elettroforesi mono- e bidimensionali, cromatografie di affinità, a scambio ionico e gel filtrazione, western blot) e per il loro **labelling con *probes* fluorescenti e di Spin elettronico**

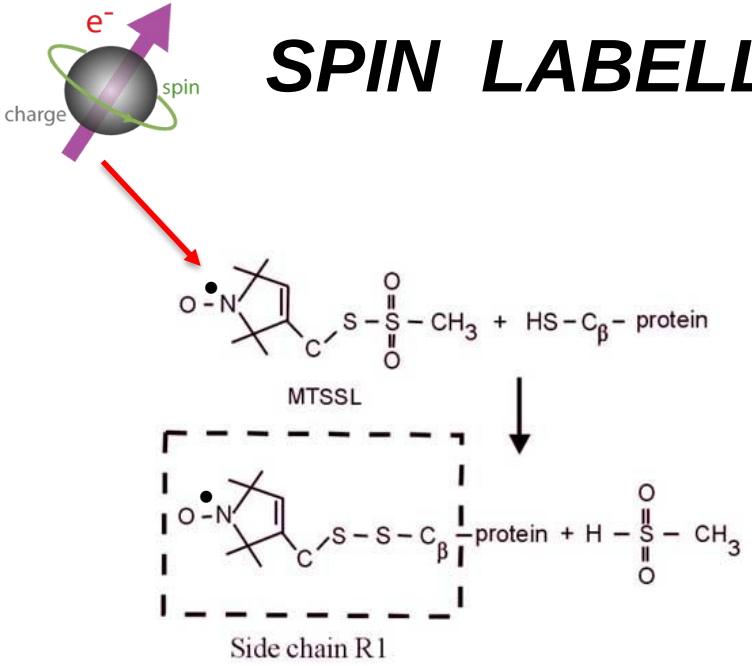
- **Metodologie Spettroscopiche** per la caratterizzazione strutturale di proteine in soluzione principalmente **FRET, CD e EPR (electron paramagnetic resonance)**

Labelling proteins

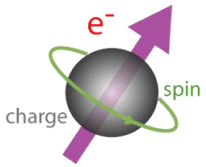
FLUORESCENT LABELLING



SPIN LABELLING

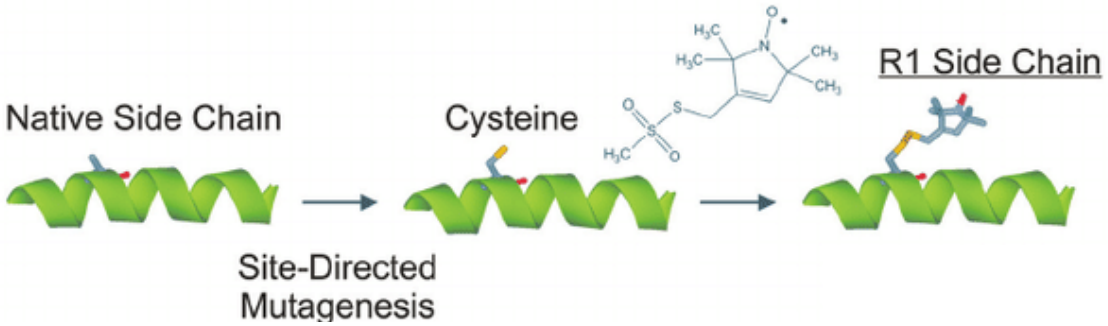


The site directed spin labelling (SDSL) Technique

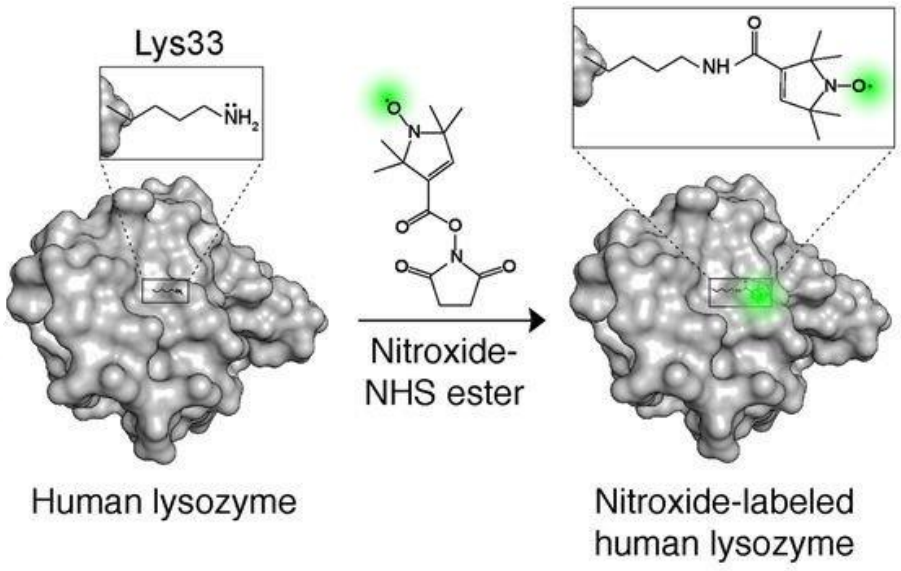


SPIN LABELING

(a)

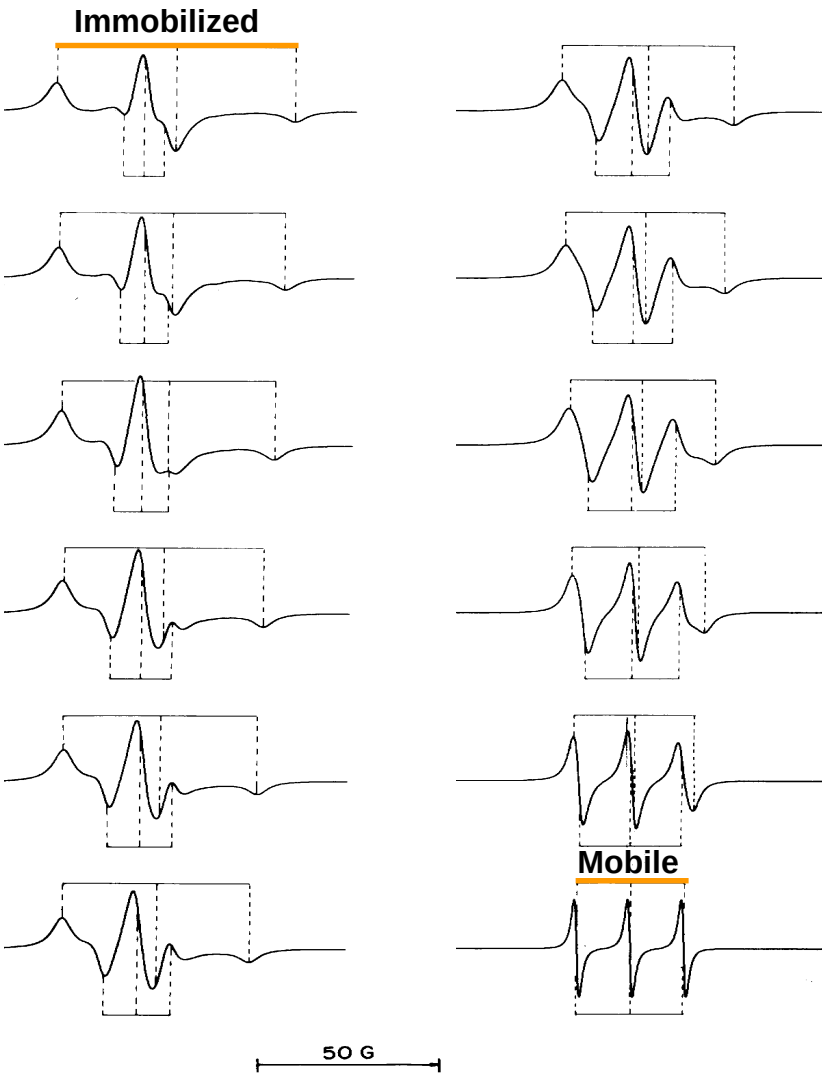


(b)

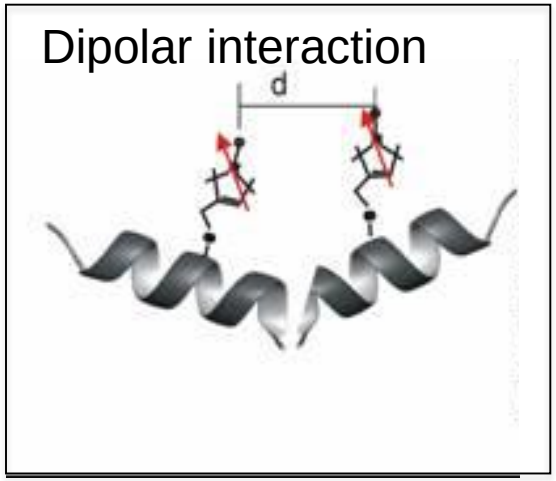


Spin labeling coupled to EPR techniques

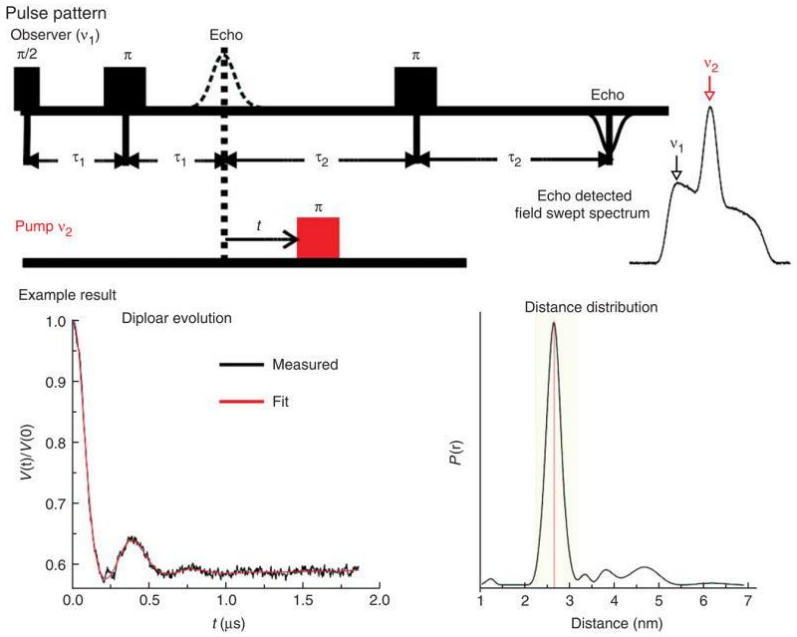
Spectral Width: Mobility parameter

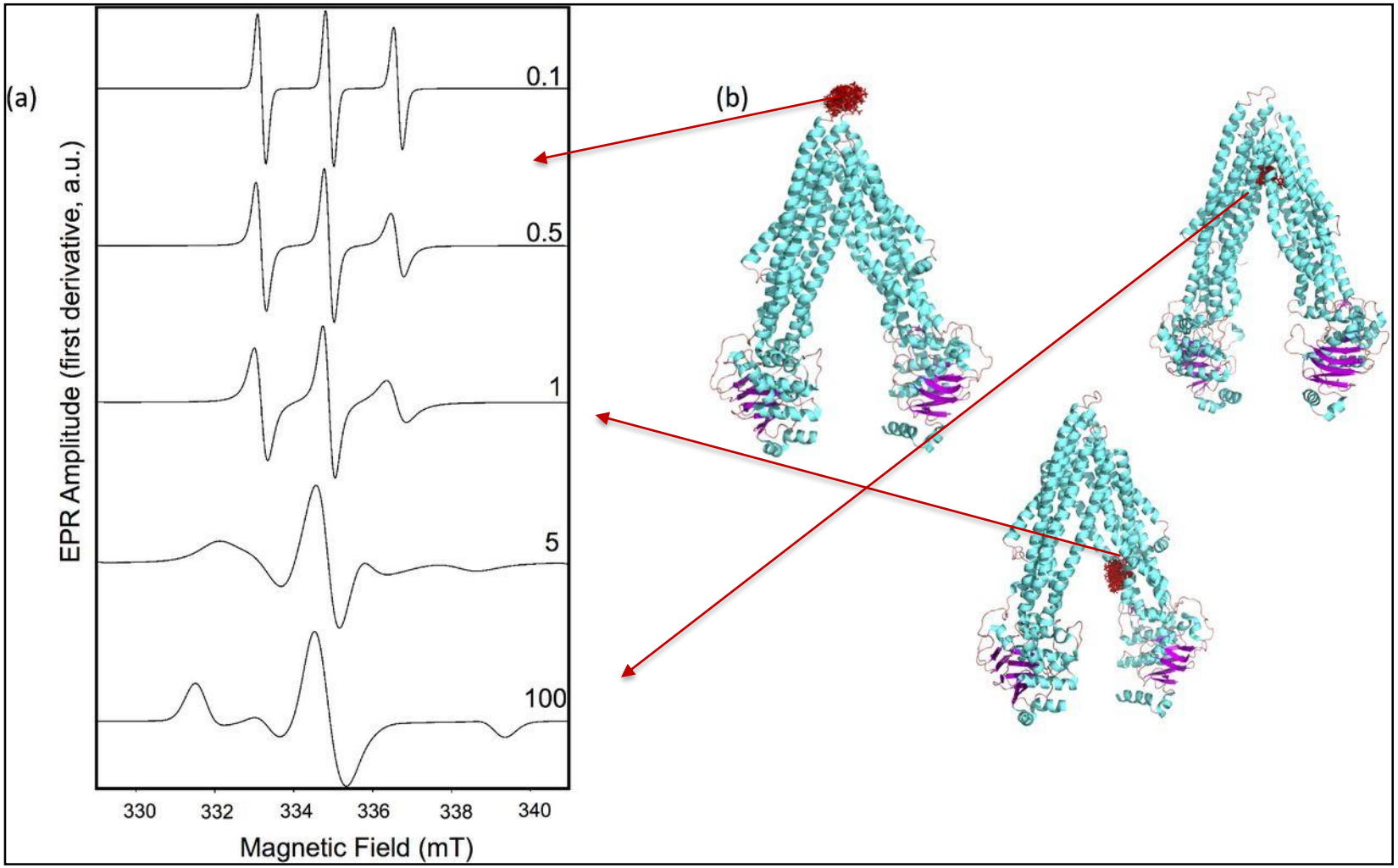


Dipolar interaction



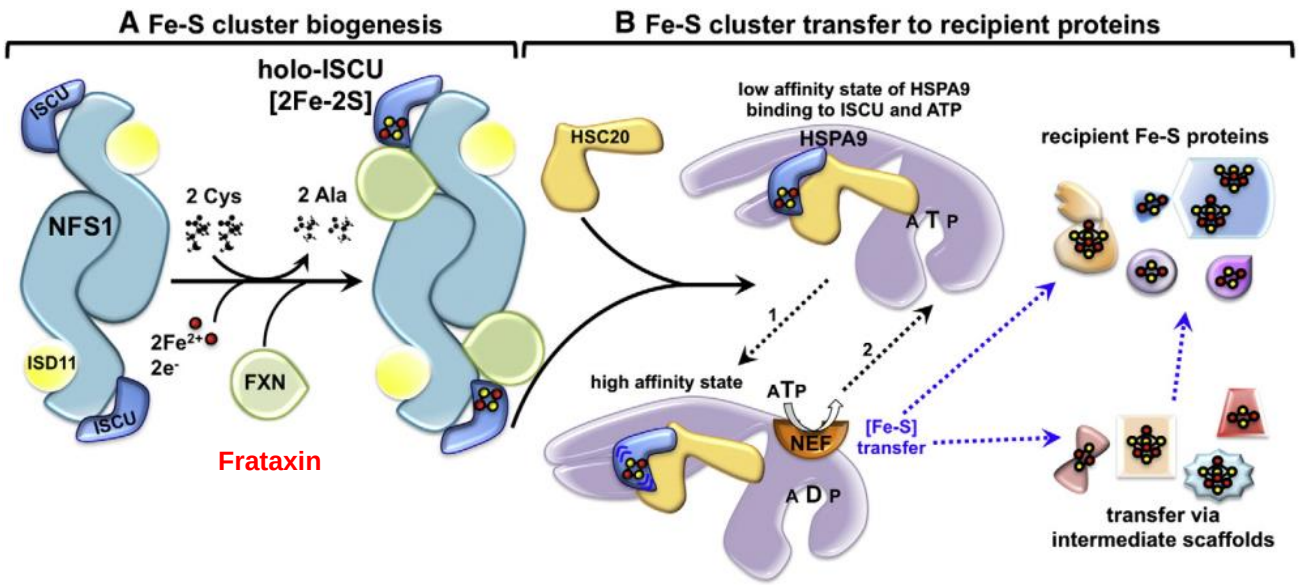
4-pulse DEER



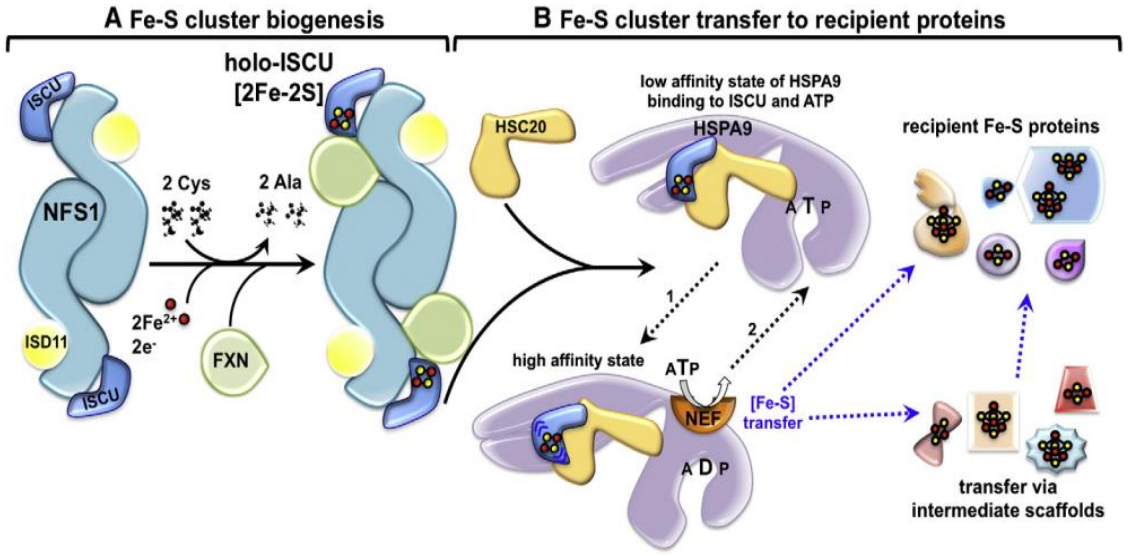

(a)
EPR Amplitude (first derivative, a.u.)
0.1
0.5
1
5
100
330
332
334
336
338
340
Magnetic Field (mT)
(b)

Assemblaggio dei centri ferro-zolfo

Frataxin, a protein involved in the iron-sulphur clusters synthesis



Frataxin, a protein involved in the iron-sulphur clusters synthesis

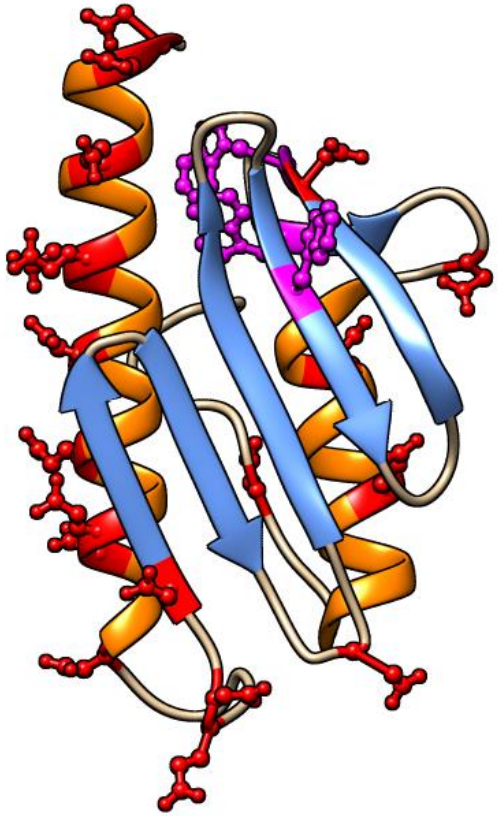


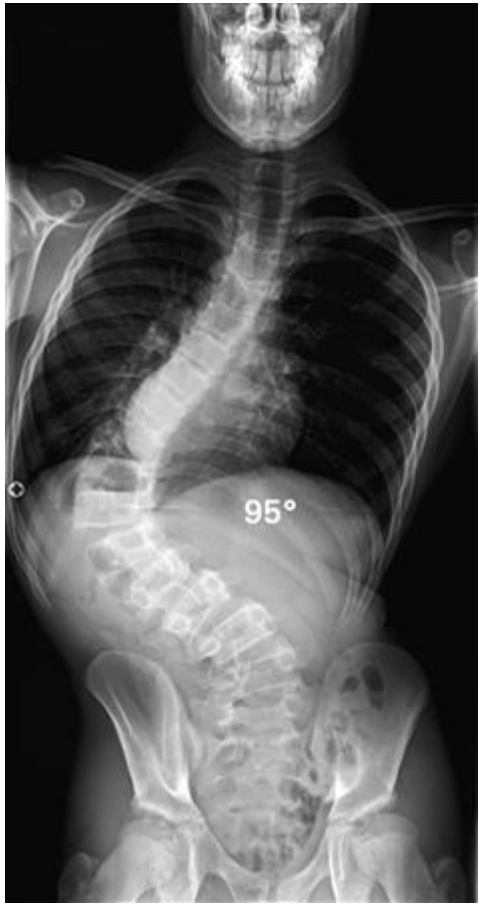
Frataxin is a small acidic protein, highly conserved in most organisms from bacteria to mammalians, which so far has been supposed to have a role in the iron-sulphur cluster biosynthesis.

A low expression level of this protein is associated to the **neurodegenerative disease Friedreich's ataxia (FRDA)**, whose main biochemical feature is a large depletion of proteins relying on iron-sulphur clusters for function

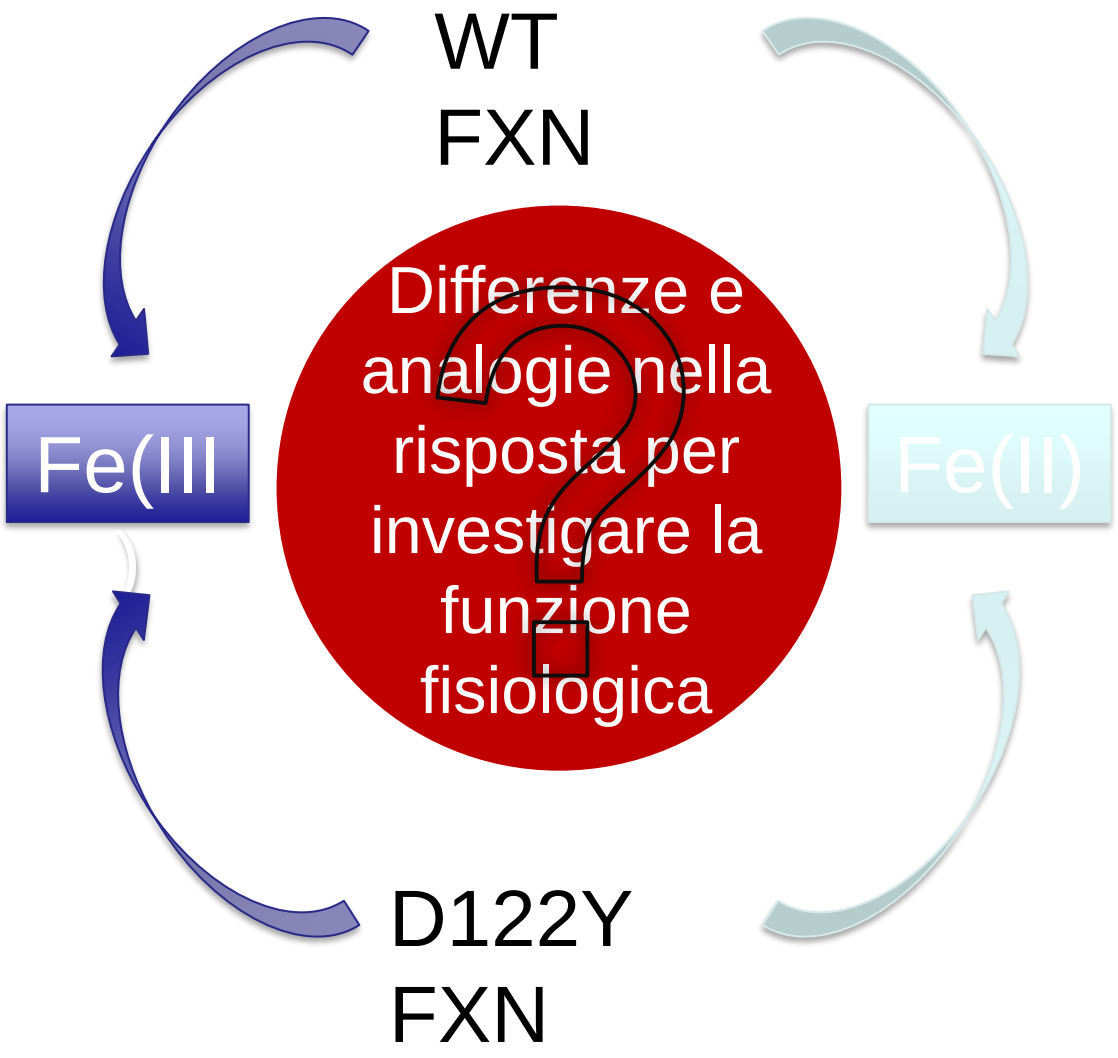
Frataxina e atassia di Friedriech

Struttura di frataxina (FXN)





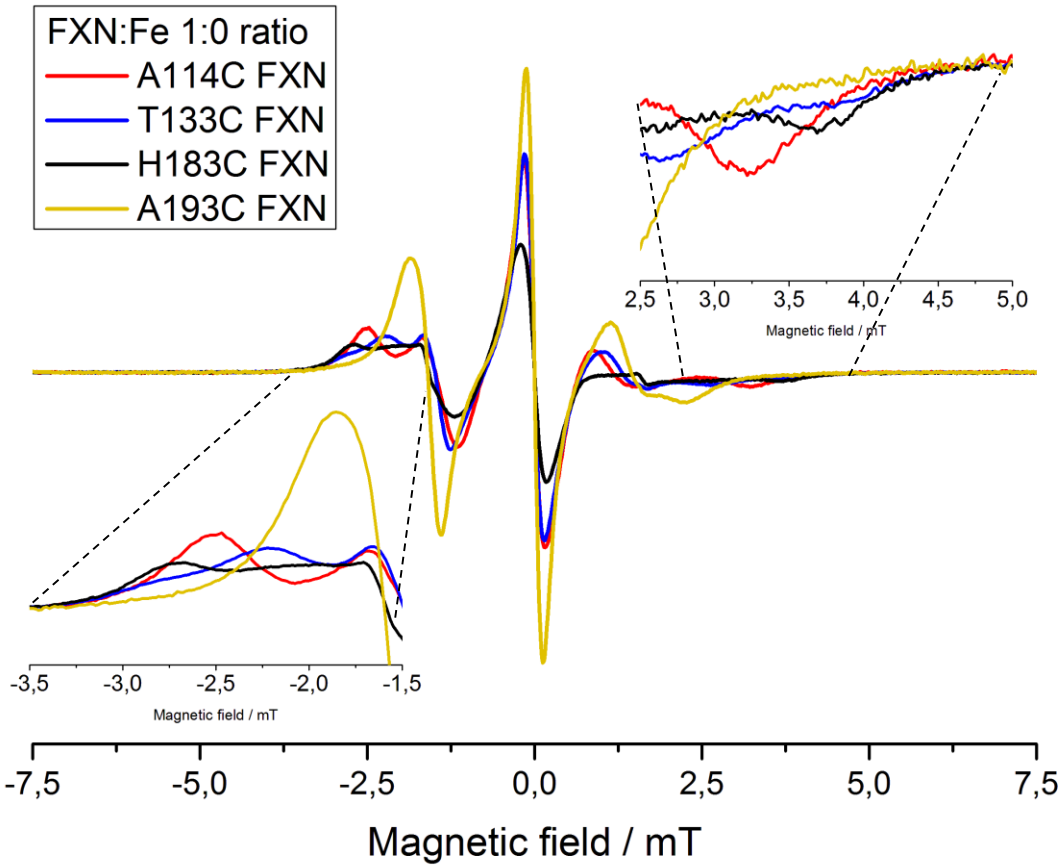
Paziente con sindrome di Friedriech

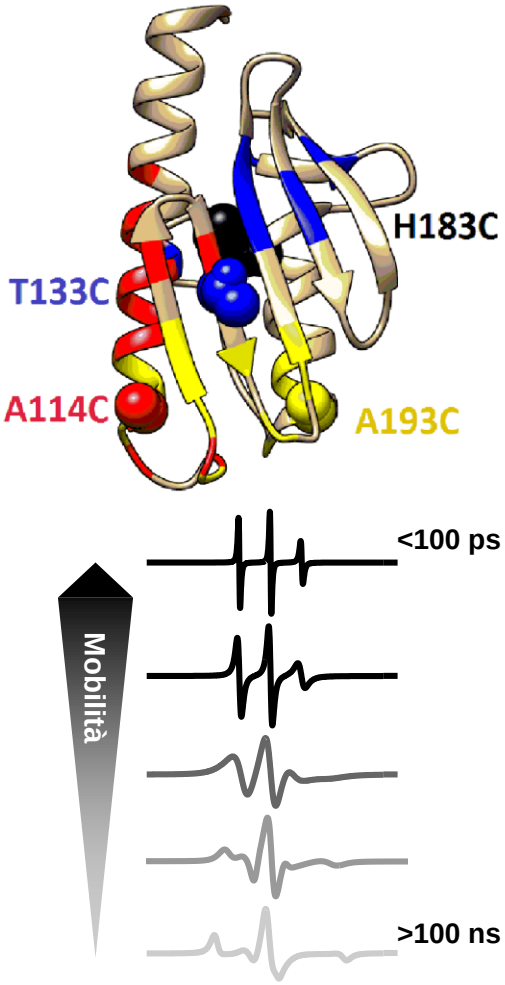


Tecniche spettroscopiche:

- Fluorescenza
- Dicroismo circolare
- Dynamic light scattering
- ***SDSL-EPR***

SDSL-EPR dei mutanti FXN-Cys

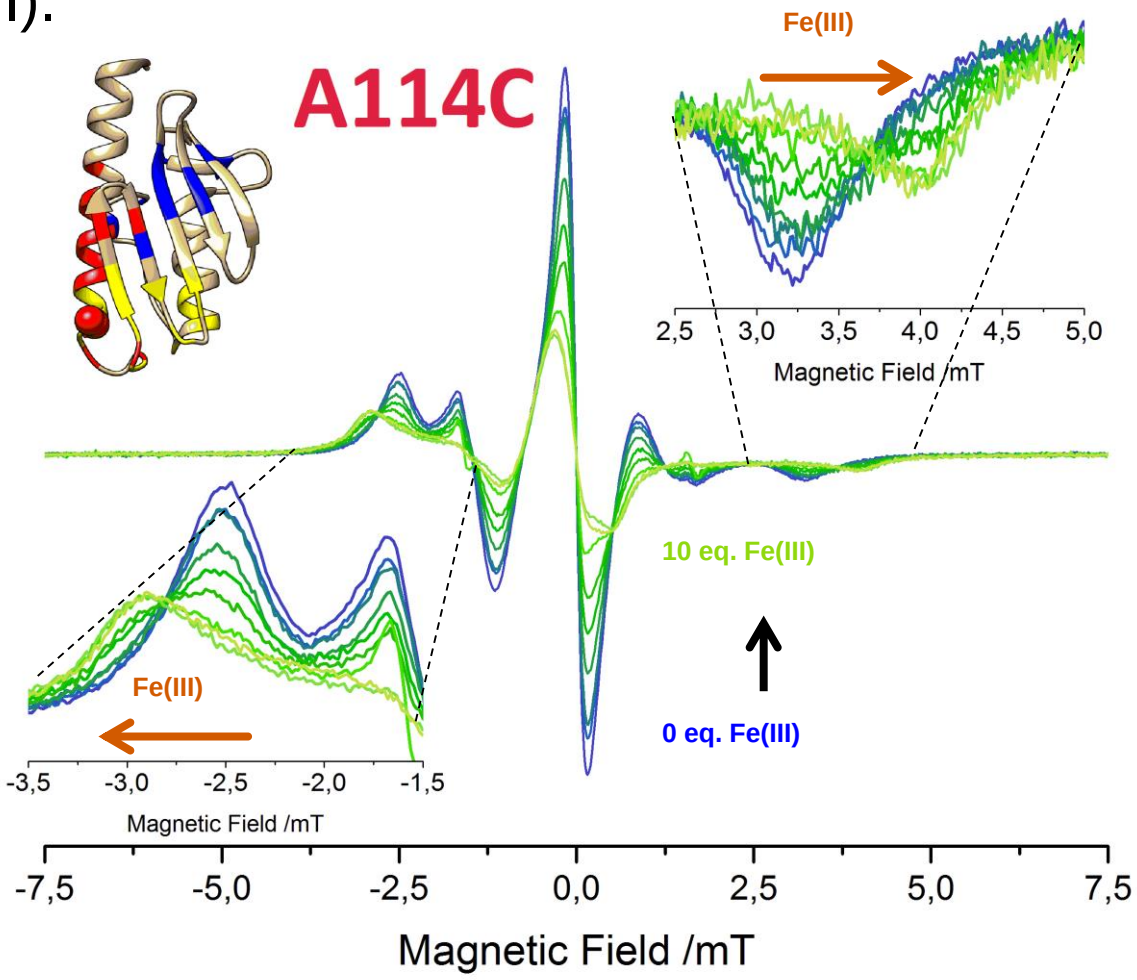


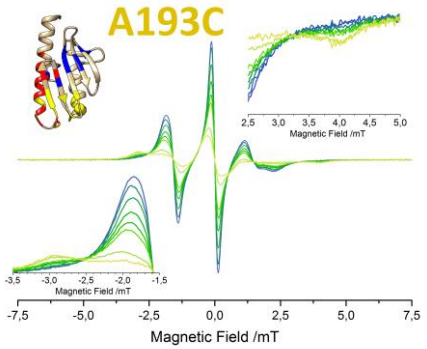


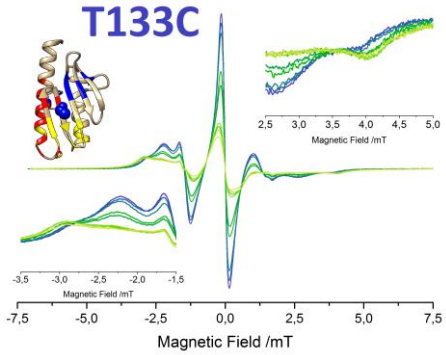
I diversi siti marcati della proteina presentano una diversa mobilità.

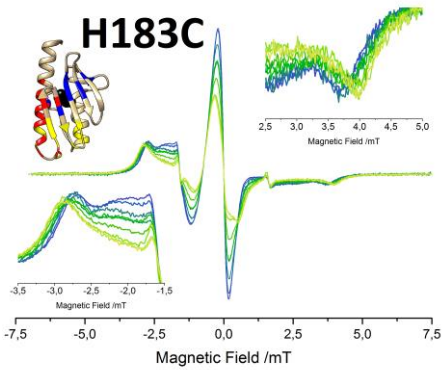
Fe(III): effetto sugli spettri EPR

Tutti i siti marcati si immobilizzano in presenza di Fe(III).

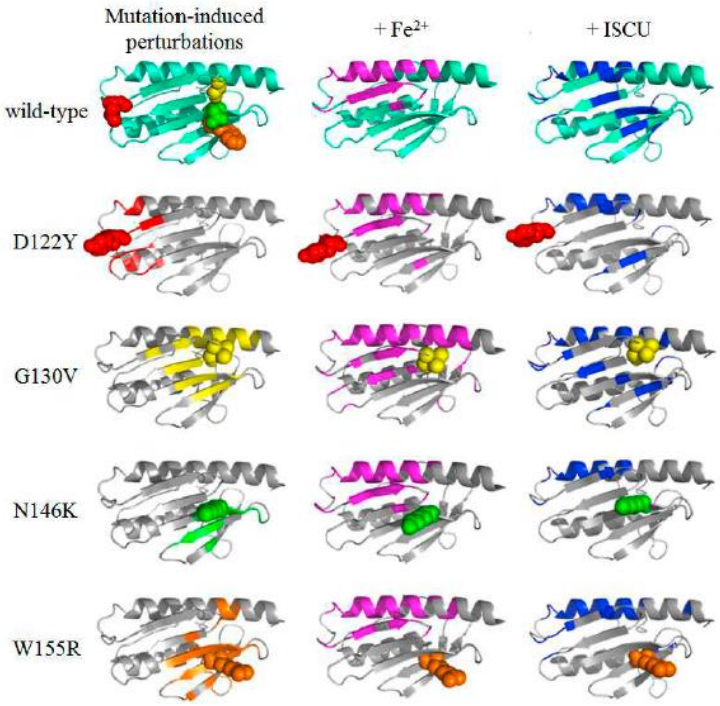






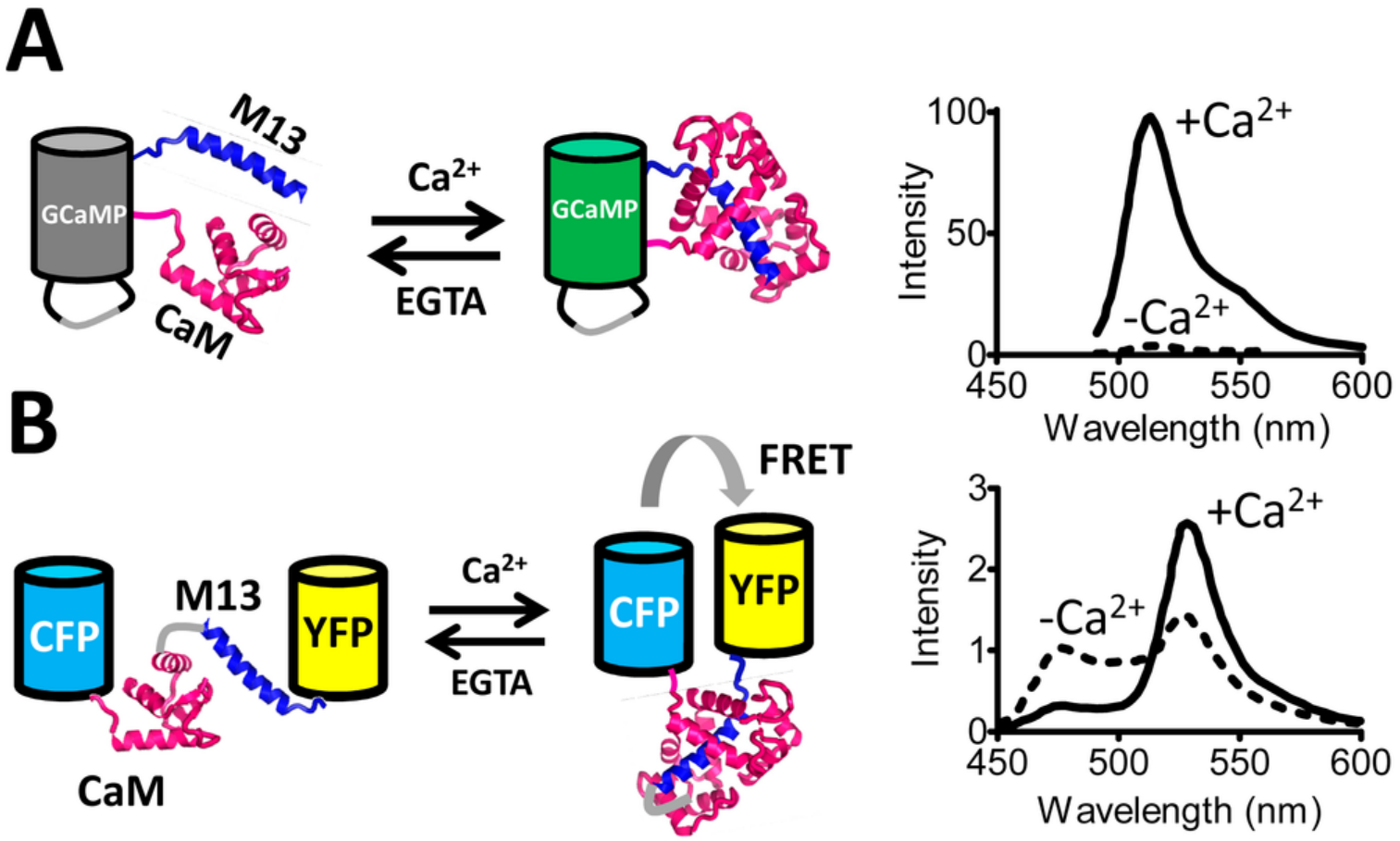


T1. In vitro experiments using purified recombinant proteins /WT and mutants) to study the conformational effects induced by Fe and the putative interface regions between FXN and ISU by FRET and EPR.



The **FXN point mutants D122Y, G130V, N146K, and W155R** are all associated with an FRDA phenotype. We have already investigated them by NMR spectroscopy (submitted), finding perturbation of the NMR spectrum in each mutant when **iron and ISU** were subsequently added.

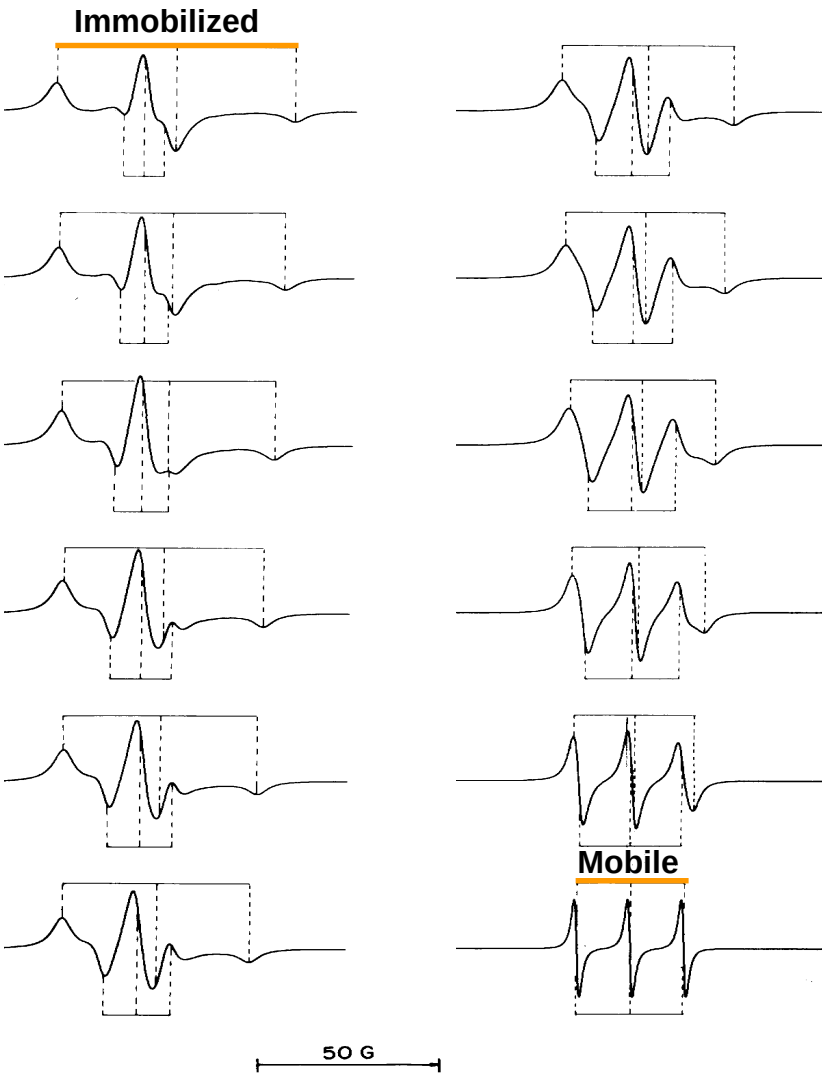
Cameleon (protein): sensori del Calcio



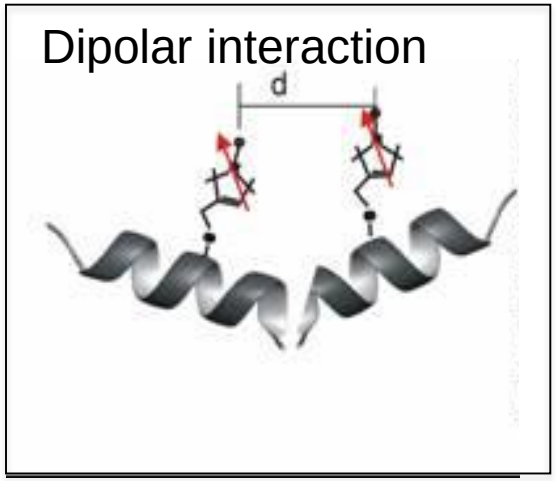
(**M13**: from skeletal muscle myosin light- chain kinase (MLCK) .

Spin labeling coupled to EPR techniques

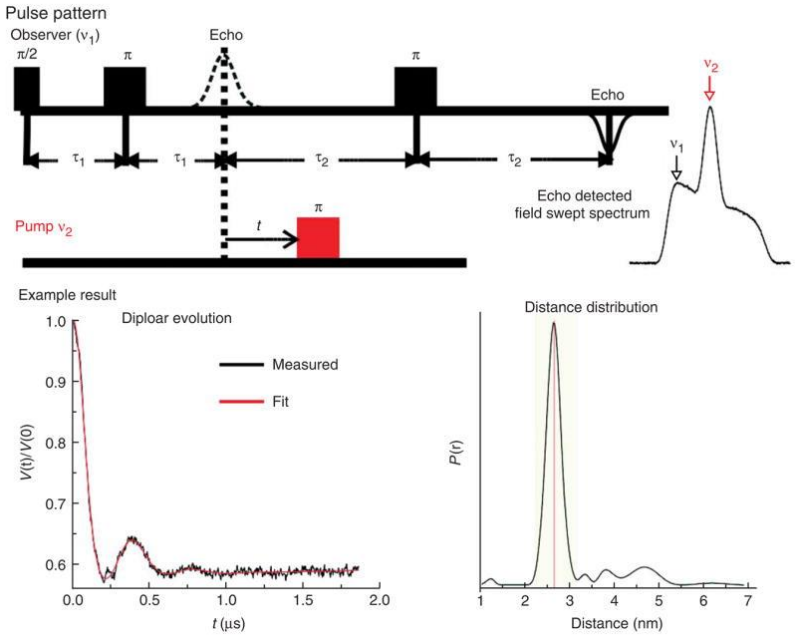
Spectral Width: Mobility parameter



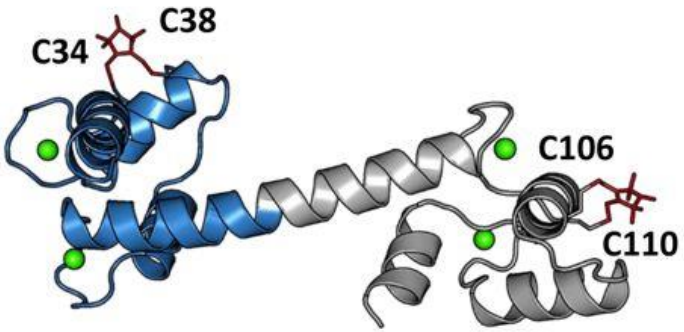
Dipolar interaction



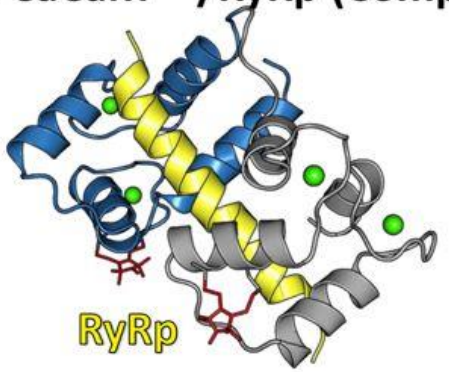
4-pulse DEER



a: CaCaM** (Open)

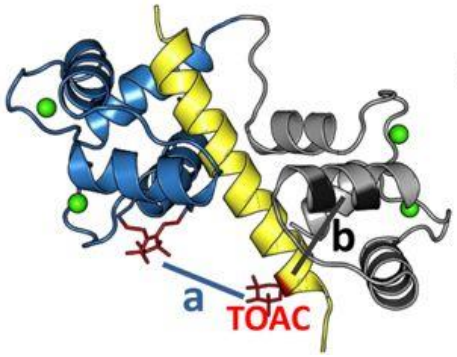


c: CaCaM**/RyRp (Compact)



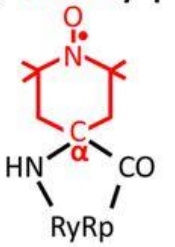
Lobe-to-Lobe Distance

e: CaCaM*/RyRp* (Compact)

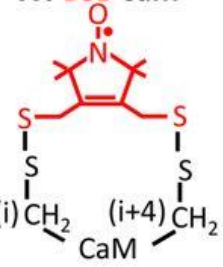


Lobe-to-RyRp Distance

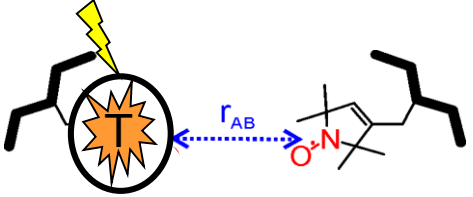
g: TOAC-RyRp



h: BSL-CaM

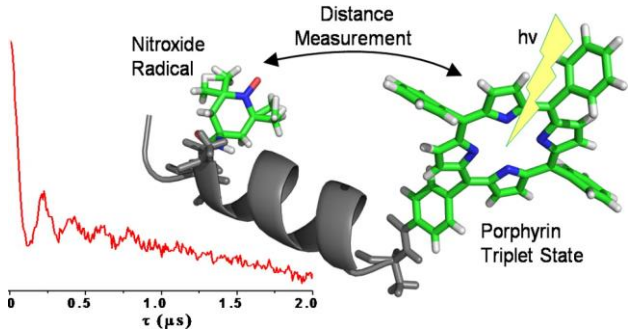


Photoexcited **triplet state – nitroxide** distance

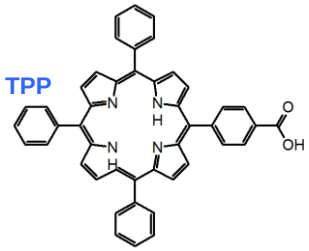


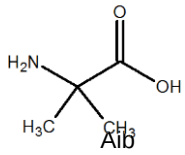
Porphyrin **Triplet State** as a Potential Spin Label for Nanometer Distance Measurements by PELDOR

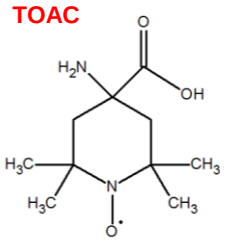
Marilena Di Valentin; Marco Albertini; Enrico Zurlo; Marina Gobbo; Donatella Carbonera; *J. Am. Chem. Soc.* **2014,** 136, 6582-6585.

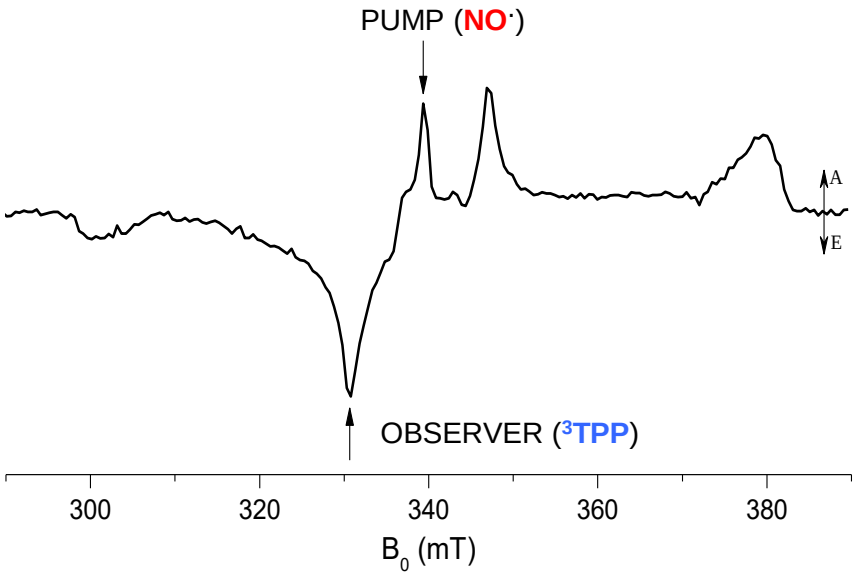


bis-labeled peptide ***TPP***-(Ala-Aib)4-Ala-***TOAC***-Ala-(Aib-Ala)2-OH.



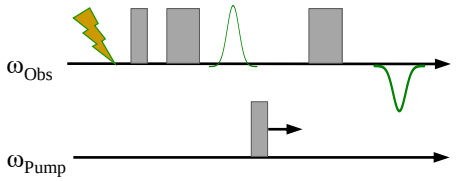






TTP-NO labeled peptides

PELDOR Pulse sequence



Photoexcited **triplet state – nitroxide** distance

TPP-(Ala-Aib)$_2$-Ala-Ala-TOAC-(Ala-Aib)$_4$-Ala-OH

18 Å

TPP-(Ala-Aib)$_2$-Ala-Aib-Ala-TOAC-(Ala-Aib)$_3$-Ala-OH

21 Å

TPP-(Ala-Aib)$_2$-Ala-Aib-Ala-Aib-Ala-TOAC-(Ala-Aib)$_2$-Ala-OH

23 Å

TPP-(Ala-Aib)$_3$ -Ala-Aib-Ala-(Ala-Aib)$_2$-Ala-TOAC-Ala-Aib-Ala-OH

31 Å

TPP-(Ala-Aib)$_2$-Ala-Aib-Ala-Aib-Ala-(Ala-Aib)$_4$-Ala-TOAC-Ala-Aib-Ala-OH

38 Å

TPP-(Ala-Aib)$_6$-Ala-Aib-Ala-Aib-Ala-Aib-Ala-Aib-Ala-Ala-TOAC-Ala-Aib-Ala-OH

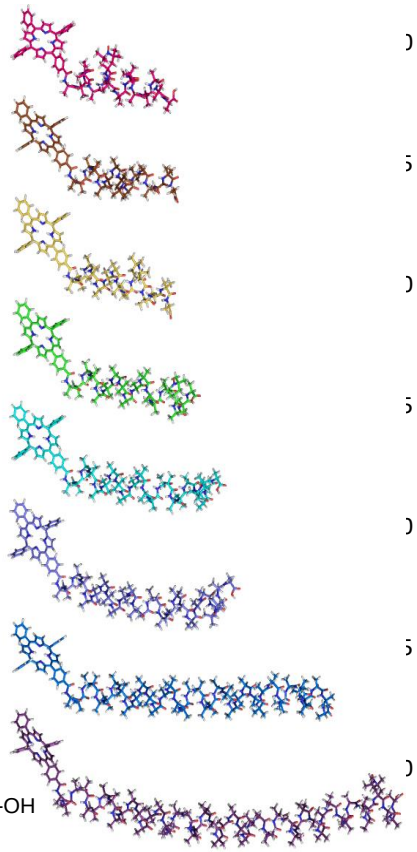
43 Å

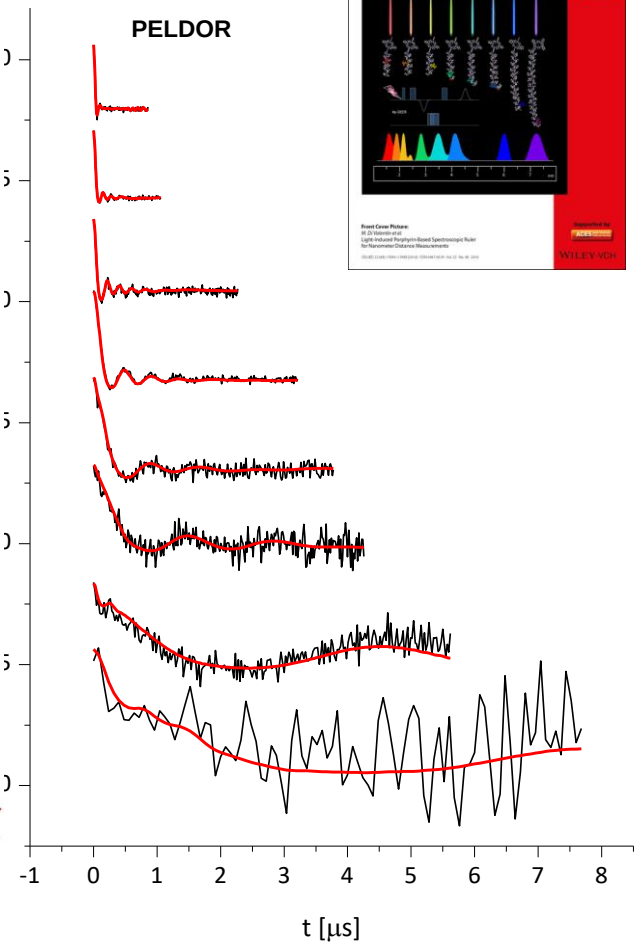
TPP-(Ala-Aib)$_6$-Ala-(Aib-Ala)$_6$-Ala-(Ala-Aib)$_2$-Ala-Aib-Ala-Aib-Ala-TOAC-Ala-Aib-Ala-OH

62 Å

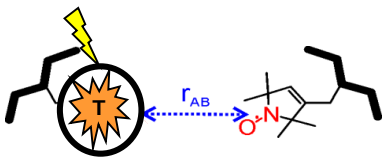
TPP-(Ala-Aib)$_{12}$-Ala-(Aib-Ala)$_6$-Ala-Aib-Ala-Aib-Ala-Aib-Ala-Aib-Ala-Ala-TOAC-Ala-Aib-Ala-OH

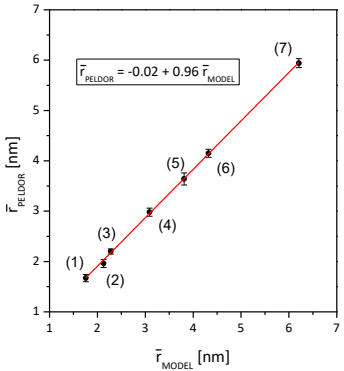
81 Å





Correlation between structural models and PELDOR derived distances





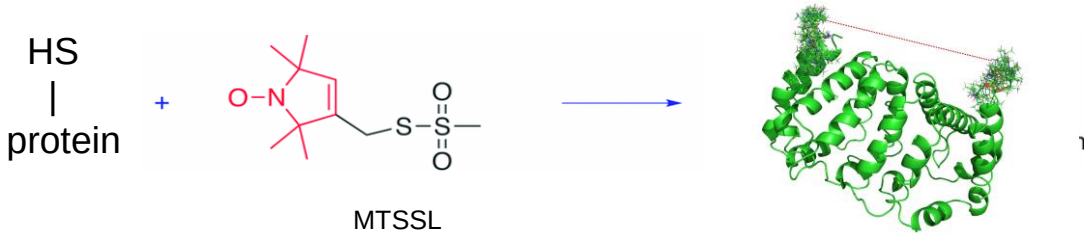
$$\hat{\mathcal{H}}_S = \omega_T \, \hat{S}_{T,z} + \omega_R \, \hat{S}_{R,z} + D \, (\hat{S}^2_{T,z} - \frac{1}{3} \, \hat{S}^2_T) + \omega_{dd} \, \hat{S}_{T,z} \, \hat{S}_{R,z}$$

$$\omega_{dd} = \frac{\mu_0 \, \mu_e^2}{4\pi \, \hbar} \, g_T \, g_R \frac{1 - 3 \, \cos^2\theta_{TR}}{r^3_{TR}}$$

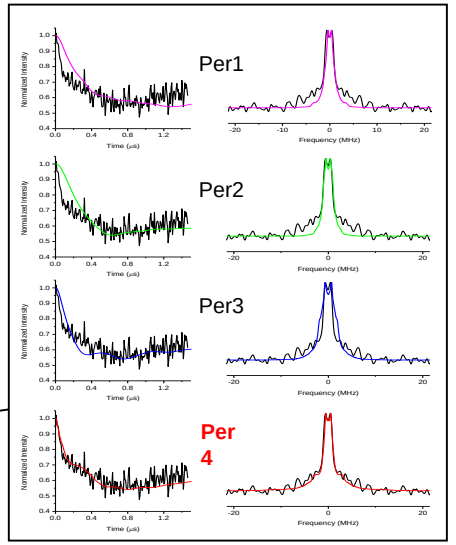
$$\omega_{dd} = \frac{\mu_0 \, \mu_e^2}{4\pi \, \hbar} \, g_T \, g_R \sum_j \rho^j_T \frac{(1 - 3 \, \cos^2\theta^j_{TR})}{\left(r^j_{TR}\right)^3}$$

Distance measurements in **peridinin-chlorophyll *a*-protein** by light-induced PELDOR spectroscopy. Analysis of triplet state localization

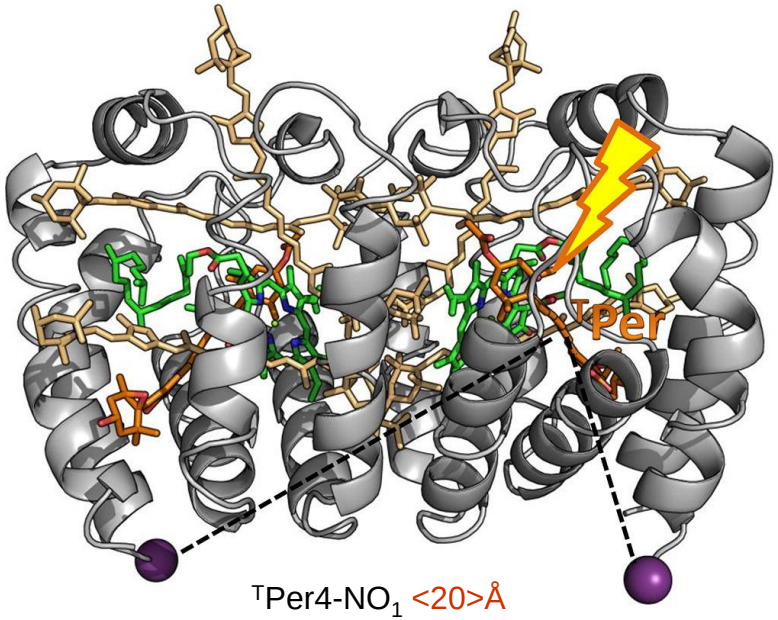
Biochimica et Biophysica Acta (BBA) – Bioenergetics ***2016*** *1857, 1909-1916*



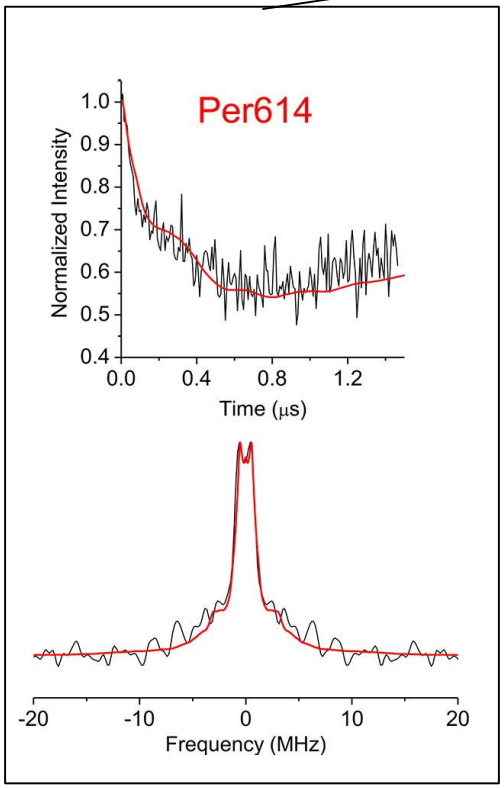
Site-directed Spin-labelling (SDSL)

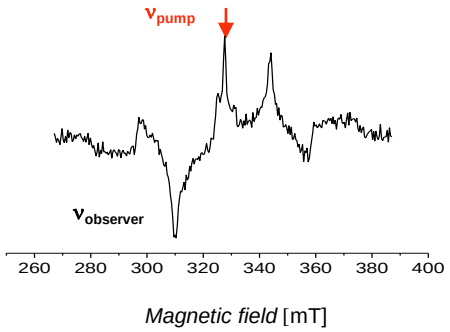


N-RFPCP (N-ter refolded PCP)



^{T}Per4-NO_1 <20>Å
^{T}Per4-NO_2 <40>Å





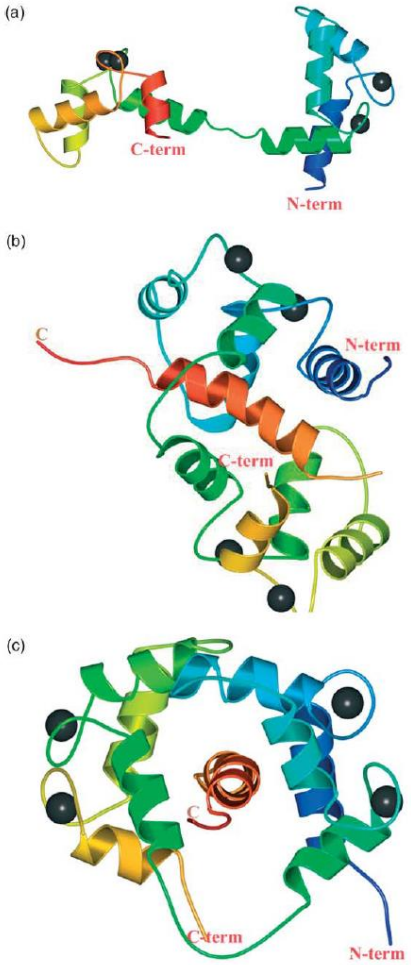
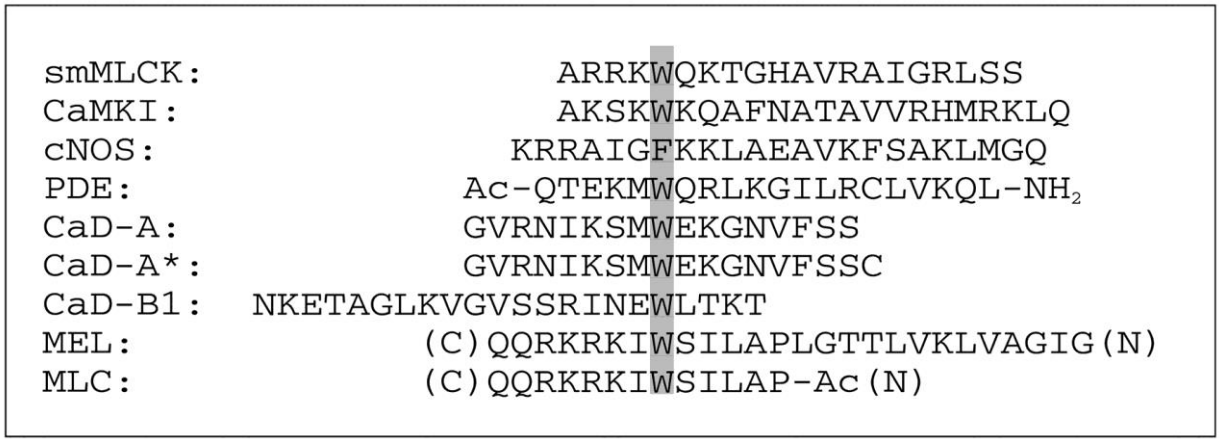


Table 1. Summary of several amino acid sequences of calmodulin binding peptides and protein target sites in part adopted from Crivici & Ikura.[3]

SkMLCK (M13)	K R R **W** K K N **F** I A **V** S A A N **R** **F** K K I S S
smMLCK	A R R K **W** Q K T G H A **V** R A I G **R** **L** S S
CaMK II	A R R K **L** K G A **I** L T T M **L** A T **R** N F S
Caldesmon	G **V** R N I K S M **W** E K G N V **F** S S
Calspermin	A R R K **L** K A A **V** K A **V** V A S S **R** **L** G S
PFK	F M N N W E V **Y** K L L **A** H I R P P A P **K** S G S Y T V
Calcineurin	A R K E V **I** R N K **I** R A **I** G K M A **R** **V** F S V L R
Ca^{2+} ATPase	R G Q I L **W** F R G **L** N R **I** Q T Q I **K** **V** V N A F S
PDE	R R K H **L** Q R P **I** F R **L** R C L V **K** Q L E K
NOS	K R R A I G **F** K K L **A** E A **V** K F S A **K** **L** M G Q
Neurom.	K A H K A A T K **I** Q A S **F** R G H I T R K **K** **L** K G E K K
Spektrin	K T A S P **W** K S A R L M **V** H T V A T **F** N S I K E
MARKS	K K K K K R **F** S F K K S F K L S G F S **F** K K S K K
Mastoparan	I N L K **A** L A **A** L A K K I **L**
Mellitin	G I G G A V **L** K V L T T G **L** P A L I S **W** I K R K R

The sequences are aligned by visual inspection of the putatively conserved major hydrophobic anchors, marked in bold and blue, and minor hydrophobic anchors marked in bold and green. Further criteria for the alignment are the putatively conserved basic residues, which are marked in black and bold. These basic residues were found in two calmodulin peptide structure[19,20] to be essential for the binding of CaM to these peptides. The hydrophobic anchors interact with hydrophobic patches of calmodulin in the structures published.[10,19,20]

Alignment of the CaM-binding peptides used in this study, as well as smMLCKp (19).



Richard D. Brokx et al. J. Biol. Chem. 2001;276:14083-14091

TABLE I
Thermodynamics of CaM-peptide interactions

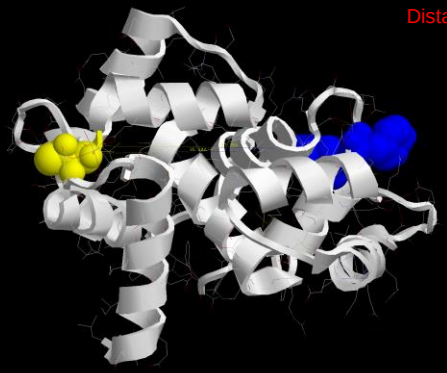
ND, not determined.

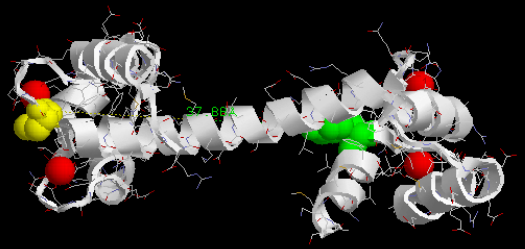
Peptide	Temperature	Buffer	N_b	ΔH_{cal}	K_a
	°C			*kJ/mol*	M^{-1}
CaMKI	5.3	PIPES	0.90	18.8	$(8.0 \pm 4.0) \times 10^7$
	10.0	PIPES	0.90	3.3	$(1.6 \pm 1.4) \times 10^7$
	15.0	PIPES	1.00	−15.1	$(2.8 \pm 0.6) \times 10^7$
	20.0	PIPES	1.10	−33.1	$(2.3 \pm 0.3) \times 10^7$
	25.0[a]	PIPES	0.93	−50.2	$(3.4 \pm 0.8) \times 10^7$
	25.0	Imidazole	1.03	−49.4	$(7.0 \pm 1.0) \times 10^7$
	25.0	Imidazole	1.01	−52.3	$(2.4 \pm 0.3) \times 10^7$
	25.0	NaCacodylate	0.80	−51.0	$(6.0 \pm 1.0) \times 10^7$
	25.0	NaCacodylate	0.75	−49.0	$(4.0 \pm 0.9) \times 10^7$
	25.0	MOPS	0.93	−50.6	$(3.2 \pm 0.7) \times 10^7$
cNOS	5.2	PIPES	0.95	60.7	$\sim 2.4 \times 10^8$
	10.0	PIPES	0.92	43.9	$\sim 1.8 \times 10^8$
	15.0	PIPES	0.99	24.3	$\sim 1.7 \times 10^8$
	20.0	PIPES	0.98	6.7	ND
	25.0[b]	PIPES	1.09	−11.7	$\sim 1.3 \times 10^8$
PDE	5.2	PIPES	0.90	51.9	$\sim 1.6 \times 10^8$
	11.0	PIPES	0.96	42.7	$\sim 1.2 \times 10^8$
	20.0	PIPES	1.00	24.7	$\sim 0.9 \times 10^8$
	24.0[c]	PIPES	0.90	15.9	$\sim 1.1 \times 10^8$
MEL	10.1	PIPES	1.00	90.0	$(3.0 \pm 1.0) \times 10^8$
	15.0	PIPES	1.08	77.8	$(1.9 \pm 0.5) \times 10^8$
	20.0[d]	PIPES	1.09	62.3	$(3.0 \pm 1.0) \times 10^8$
MLC	5.0	PIPES	0.97	31.4	$(2.3 \pm 0.4) \times 10^6$
	15.1	PIPES	0.96	16.7	$(3.8 \pm 0.5) \times 10^6$
	25.1	PIPES	ND	2.3	ND
CaD-A	5.0	PIPES	0.93	−33.1	$(4.1 \pm 0.4) \times 10^6$
	10.1	PIPES	0.90	−41.8	$(3.5 \pm 0.2) \times 10^6$
	15.0	PIPES	0.97	−49.4	$(3.3 \pm 0.3) \times 10^6$
	20.0	PIPES	0.92	−57.7	$(2.2 \pm 0.1) \times 10^6$
	25.0[e]	PIPES	0.90	−66.1	$(1.4 \pm 0.1) \times 10^6$
CaD-A*	5.0	PIPES	1.03	−24.3	$(0.8 \pm 0.4) \times 10^6$
	10.0	PIPES	1.03	−36.0	$(1.0 \pm 0.5) \times 10^6$
	17.0	PIPES	0.97	−39.3	$(1.7 \pm 0.8) \times 10^6$
	25.0	PIPES	0.96	−49.0	$(1.4 \pm 0.7) \times 10^6$
CaD-A*-CH_3	25.0	PIPES	0.90	−50.6	$(0.4 \pm 0.2) \times 10^6$

[a] $K_a = 5.0 \times 10^8\ M^{-1}$ (29).
[b] $K_a = 5 \times 10^8\ M^{-1}$ (55, 56).
[c] $K_a = 4.5 \times 10^6\ M^{-1}$ (22).
[d] $K_a = 3.3 \times 10^8\ M^{-1}$ (85).
[e] $K_a = 1.3 \times 10^6\ M^{-1}$ (24).

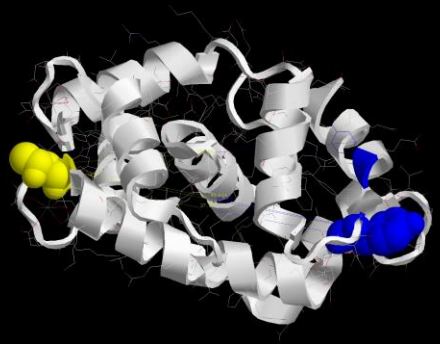
Fig. 4. Comparison of selected CaM-target sequences as observed in canonical CaM-complex structures. Anchor residues are shown in red. Further details are provided in Table 1.

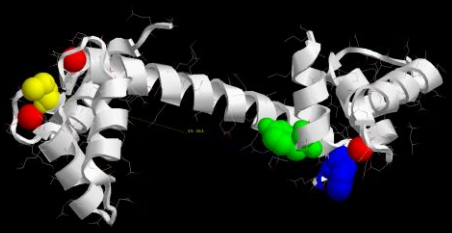
Distanza Cys-Tyr138 varia poco sempre intorno ai 35 A



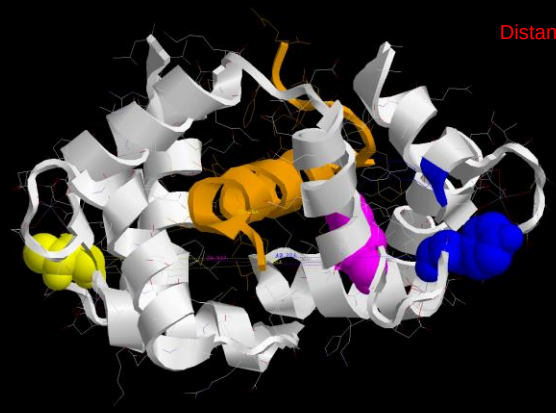


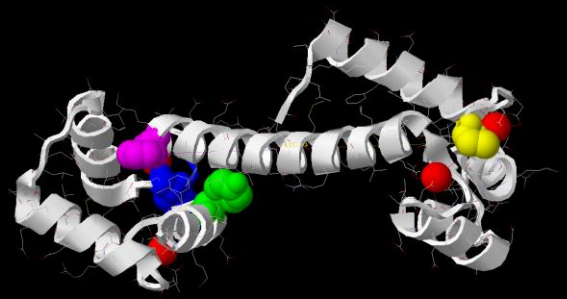
Distanza Cys-Tyr99 varia da 41 a 49 A





Distanza Cys-Phe92 varia varia da 29 a 44 A

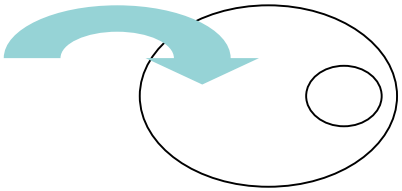




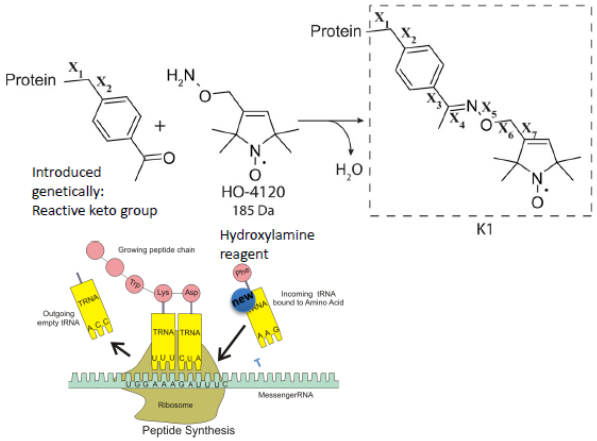
in vivo experiments

Mechanical incorporation

Labeled proteins



Unnatural aminoacids



New
side chain K1
(Fleisser et al., PNAS 2010)

(++) Can be used when functional cysteines (or disulfide bonds) are present and cannot be neither removed, nor labeled (to avoid protein denaturation, or malfunction).