Electronic Supplementary Material (ESI) for RSC Advances.

Water soluble Calcium-Sodium based co-ordination polymer: Selective Release of Calcium at Specific Binding Sites on Protein

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Supporting Information

General

All reagents and solvents for the synthesis were purchased from Sigma Aldrich Chemical Co. Pvt. Ltd., India and E. Merck respectively, and used as received. ^{15}N labeled ammonium chloride for protein preparation was purchased from Cambride Isotope Laboratories (USA) and D_2O (99.96% pure) from Sigma Aldrich. UV-visible, Infrared and luminescence spectra were recorded on Jasco UV-630 spectrophotometer, VARIAN 3100 FTIR, and Perkin Elmer LS-45 spectrophotometer, respectively. C, N, H content was determined by Carbo-Erba elemental analyzer 1108.

Synthesis of Ca/Na-1

EGTA (0.380 g, 1 mmol), $Ca(NO_3)_2.4H_2O$ (0.944 g, 4 mmol), and NaOH (0.160 g, 4 mmol) in H_2O (20 mL) were heated together at 110 °C for 24 h. The reaction mixture was then cooled to room temperature and left undisturbed. Colorless crystals were observed after two weeks. Anal. Calc. (%) for $C_{28}H_{66}N_5O_{36}NaCa_4$: C, 27.29; H, 5.40; N 5.68; Found: C, 27.46; H, 5.84; N, 5.47. IR (KBr pellet, cm^{-1}): 3430, 2934, 2426, 2070, 1635, 1435, 1384, 830, 645. ^1H NMR (90% H_2O+ 10% D_2O mixture, δ ppm): 3.8825 (t, -N-CH_2-COO-) 3.7284 (s, -OCH_2-), 3.467 [(d, -OCH_2-CH_2- J = 17.79 Hz), 3.2816 (d, -N-CH_2-COO, J = 17.79 Hz), 2.9346 (t, -NCH_2-), ^{13}C NMR (90% H_2O+ 10% D_2O mixture, δ ppm): 180.412 ($OOC_{(A)}$), 69.144 (-O-$C_{(E)}$-CH_2), 68.181 (-O-$C_{(D)}$-CH_2-N), 61.829 (-N-$C_{(B)}$-COO), 57.569 (-N-$C_{(C)}$-CH_2); ^{23}Na NMR (90% H_2O+ 10% D_2O mixture, δ ppm): -0.967. UV-vis (λ_{max} (nm) water, 10^{-5} M): 302, 354, and 388 nm. $\lambda_{emission}$: 452 nm at $\lambda_{excitation}$ at 388 nm.

Photophysical Properties

Absorption spectrum of Ca-Na-1 was recorded in water (10 mM). Ligand based peaks were observed at λmax 302, 354, and 388 nm. Former strong bands are assigned to π-π*

transitions, and latter band arises from n-π* transition of carboxylate of EGTA. An intense emission peak is displayed at λmax 452 nm on excitation at λmax 388 nm. The emission probably arises from the ligand [Ref 1].

X-ray crystallographic studies

The X-ray diffraction data were collected by mounting a single-crystal of the sample on glass fibers. Oxford diffraction XCALIBUR-EOS diffractometer was used for the determination of cell parameters and intensity data collection at 150(2) K. Monochromating Mo Kα radiation (λ = 0.71073 Å) was used for the measurements. The crystal structure was solved by direct methods and refined by full matrix least squares SHELXL-97 [Ref. 2]. Atoms were located from iterative examination of difference F-maps following least-squares refinements of the earlier models. Hydrogen atoms were placed in calculated positions and included as riding atoms with isotropic displacement parameters 1.2 Ueq of the attached C atoms. Due to high degree of hydration, thermal motion and disorder, hydrogen atoms for coordinated water molecule could not be located or fixed. The structure was examined using Addsym subroutine of PLATON [Ref. 3] to ensure that no additional symmetry could be applied to the models.

NMR spectroscopy

Uniformly ^{15}N labeled M-crystallin was over-expressed and purified as described earlier [Ref. 4]. For NMR experiments, protein sample was concentrated using amicon centrifugal filter (3 kD cut-off) to a final concentration of 1 mM. Protein concentration was estimated by measuring the absorbance at λmax 280 nm (ε = 19060 M^{-1} cm^{-1}). NMR experiments were performed on a Bruker Avance 800-MHz spectrometer, equipped with pulsed-field gradient units and triple resonance cryo-probe with actively shielded Z–gradients. 1D ^{1}H, ^{13}C and ^{23}Na spectra were recorded using 10 mM Ca/Na-1. Sensitivity enhanced [^{15}N-^{1}H] HSQC

spectra were recorded for pure protein and protein/Ca/Na-1 complex at 25 °C. The data were processed using Bruker Topsin 2.1 software provided with the instrument and analyzed by CARA. ^{1}H chemical shifts were calibrated relative to 2,2-dimethyl-2-silapentane-5-sulphonate at 25 °C and ^{15}N chemical shifts were referenced indirectly. Chemical shifts reported for M-crystallin apo (Ca^{2+} free) and holo (Ca^{2+} bound) forms, PDB 2k1x and 2k1w respectively, have been used as reference for the $^{1}H^{N}$ and ^{15}N chemical shift assignment [Ref. 5].

MALDI-TOF mass spectroscopy

Mass spectra were acquired on MALDI-TOF mass spectrometer (Bruker Daltonics Ultraflextreme, Germany). Pure Ca/Na-1 was dissolved in Tris-HCl buffer (pH 7.4) and 100 mM NaCl. For M-crystallin protein and Ca/Na-1 complexation studies, series of reaction mixtures were set up by varying the protein: Ca/Na-1 ratio (1:0.5, 1:1, 1:2, and 1:3) and incubated at 25 ºC for 30 min. In each case, 0.5 µl of the sample was co-crystallized with 0.5 µl of Sinapinic acid directly on the MTP ground steel target plate (Bruker). MALDI mass spectra were recorded in linear positive mode within m/z range of 5-20 kD using Bruker smartbeam-II laser system with a fine setting of 70%. Each spectrum was obtained by averaging 250 individual laser shots and was externally calibrated using peptide calibration standard (Bruker) for pure compound and protein calibration standard 1 (Bruker) for complexation. The data were analyzed on Flex analysis software (Bruker Daltonics).

Field emission scanning electron microscopy

M-crystallin and Ca/Na-1 in 1:3 molar ratio were incubated at room temperature for 3 hours to ensure completion of reaction. Following this, protein and co-ordination polymer were then separated by passing the reaction mixture through pre-equilibrated Biorad-P6 gel filtration column. Morphological characteristics of Ca/Na-1 post separation were analyzed by Field emission scanning electron microscope (Zeiss Ultra FEG 55) with operating voltage

ranged from 5 to 20 kV. The samples were simply coated with a thin film of Au and then analyzed for thickness and homogeneity of coverage.

Isothermal titration Calorimetry

For ITC measurements, 0.22 μM filtered solution of protein (chelex treated) and Ca/Na-1 were degassed prior to titration. Each titration was carried out by injecting 3 μl aliquots of 1 mM Ca/Na-1 into 60 μM of protein. A total of 40 injections were performed with a difference of 3 minutes to correct the baseline of the titration peak. Separately, aliquots of concentrated Ca/Na-1 were injected into buffer without protein to ensure subtraction of heat of dilution. Experiments were repeated using 1 mM standard $CaCl_2$ solution as injectant. All the experiments were performed at 25 °C with identical parameters. ITC data were analyzed using ORIGIN 7.0 software supplied with the instrument and curve fitting was done to fit the amount of heat released per titration.

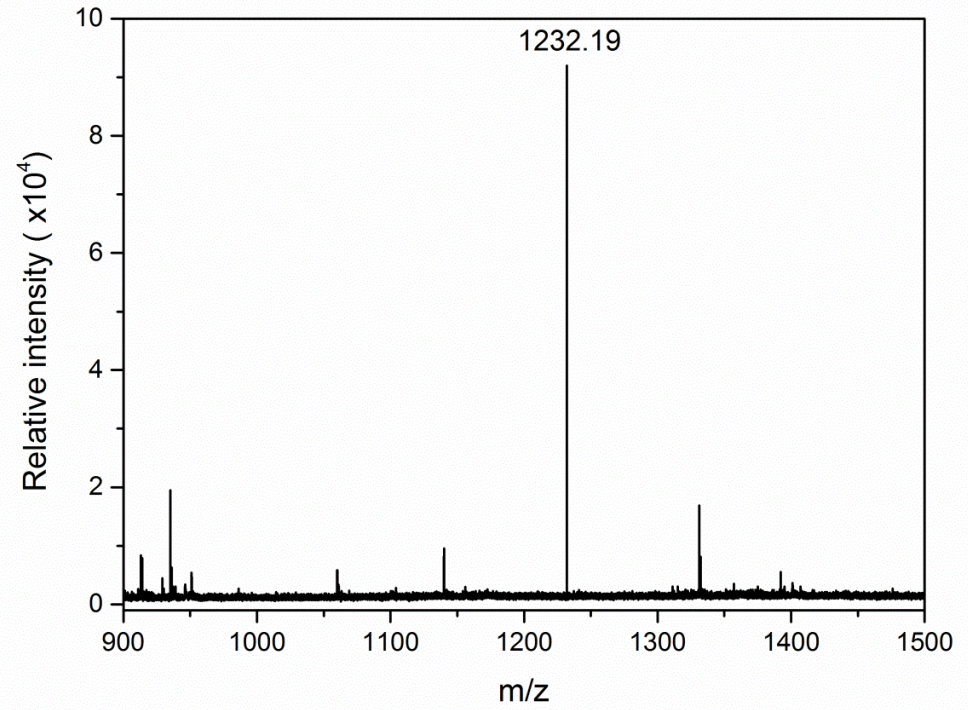
Supplementary Table 1: Crystallographic parameters of Ca/Na-1.

Parameters	*Ca/Na-1*
Chemical formula	$C_{28}H_{40}N_5O_{36}NaCa_4$
Formula weight	1205.96
Temperature (K)	150(2)
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	*P21/c*
a(Å)	19.304(3)
b(Å)	16.8700(14)
c(Å)	19.321(3)
α(º)	90
β(º)	107.10(2)
γ(º)	90
Volume(Å^3)	6013.9(14)
Z	4
Absorption coefficient (mm^{-1})	0.455
F(000)	867
Theta range for data collection (º)	3.24 to 28.97
Reflections collected / unique	15926 / 15926 [R(int) = 0.0856]
Completeness to theta %	98
Goodness-of-fit on F^2	0.894
Final *R* indices [$I>2\sigma(I)$]	*R1 = 0.0980, wR2 = 0.2245*
R indices (all data)	*R1 = 0.1674, wR2 = 0.2672*
Largest diff. peak and hole (e.Å^3)	1.722 and -0.992

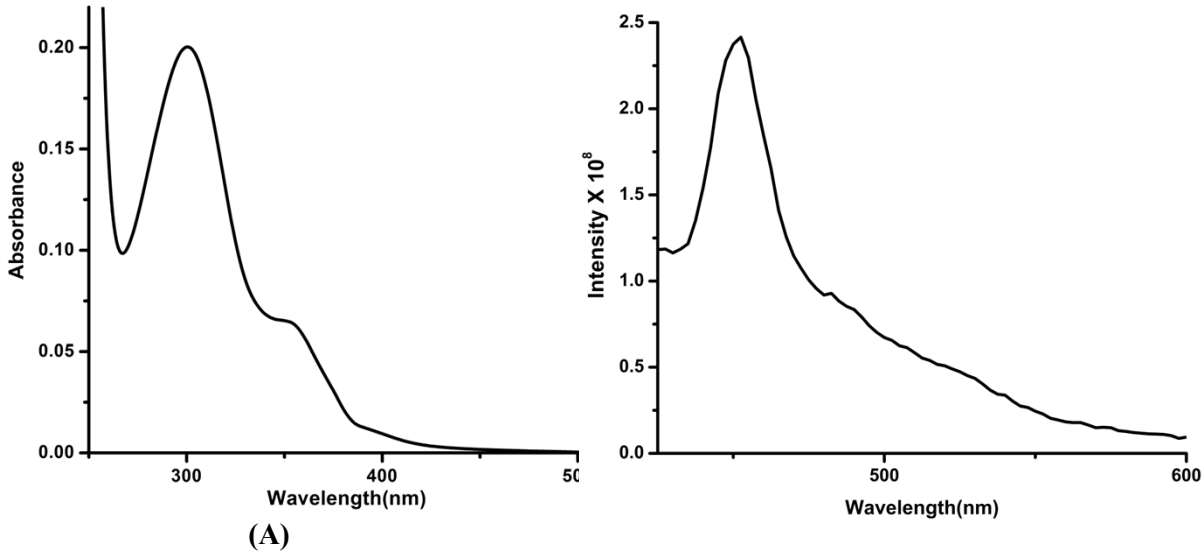
Supplementary Table 2: Selected bond lengths (Å) and bond angles (°) of Ca/Na-1.

O(46)-Na(5)	2.272(9)	C(034)-O(22)-Ca(1)	126.4(5)
O(3)-Ca(2)	2.487(5)	O(26)-Ca(1)-O(30)	131.92(17)
Ca(4)-O(12)	2.510(5)	O(27)-Ca(4)-O(11)	83.91(18)
Ca(4)-O(26)	2.541(5)	O(9)-Ca(4)-O(6)	81.3(3)
Ca(4)-O(11)	2.560(5)	O(9)-Ca(4)-O(10)	74.0(3)
Ca(4)-O(7)	2.458(5)	O(9)-Ca(4)-O(7)	78.9(3)
Ca(1)-N(4)	2.623(5)	C(18)-O(46)-Na(5)	150.5(10)
O(36)-Ca(2)	2.440(5)	O(25)-Ca(1)-N(4)	81.7(2)
N(4)-C(025)	1.473(9)	N(4)-Ca(1)-N(3)	143.52(19)
C(005)-N(2)	1.471(11)	O(34)-Ca(2)-C(035)	104.1(2)
C(015)-C(026)	1.541(10)	N(2)-C(005)-C(054)	109.7(7)

Supplementary Figure 1: MALDI-TOF mass spectrum of Ca/Na-1 in Tris-HCl buffer (pH 7.4) and 100 mM NaCl.

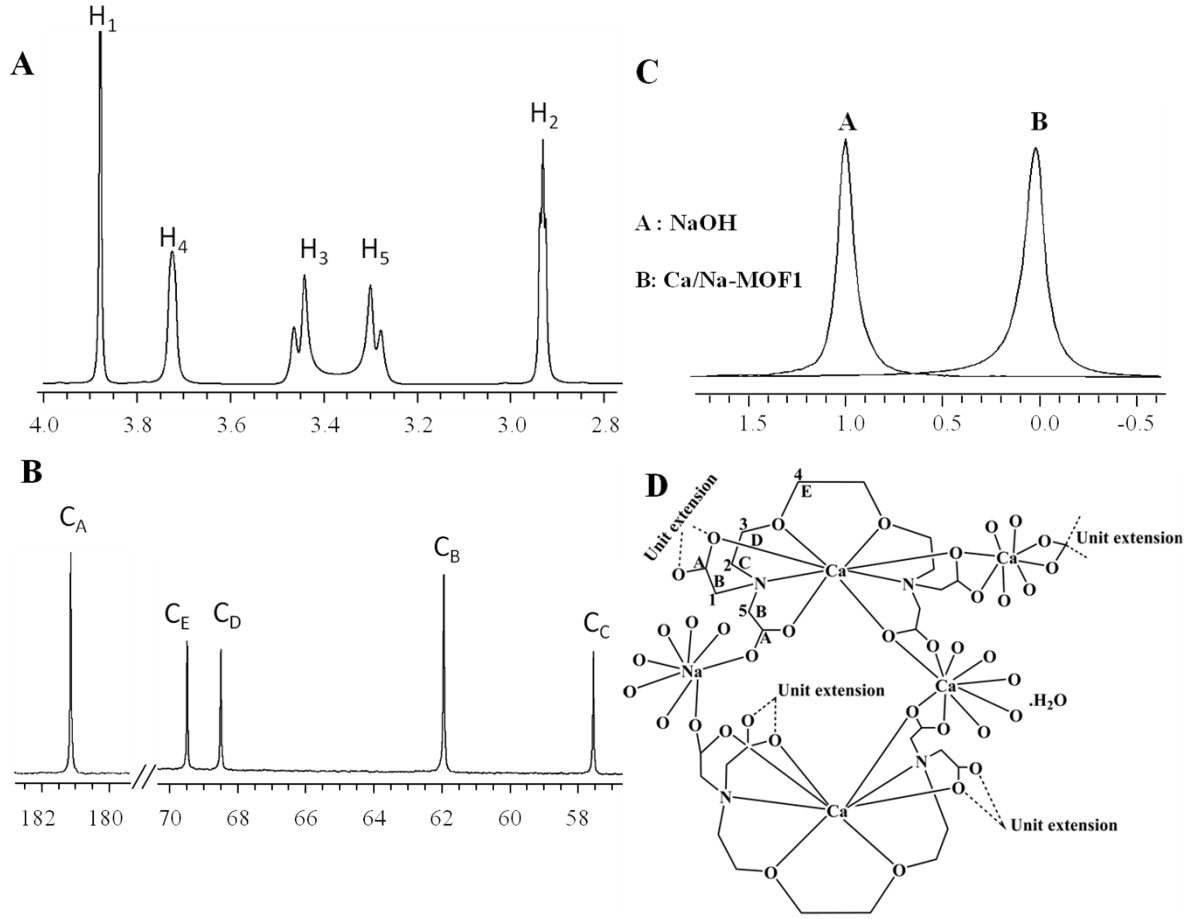


Supplementary Figure 2: UV-visible spectrum (A) Emission spectrum (B) of Ca/Na-1 in water.

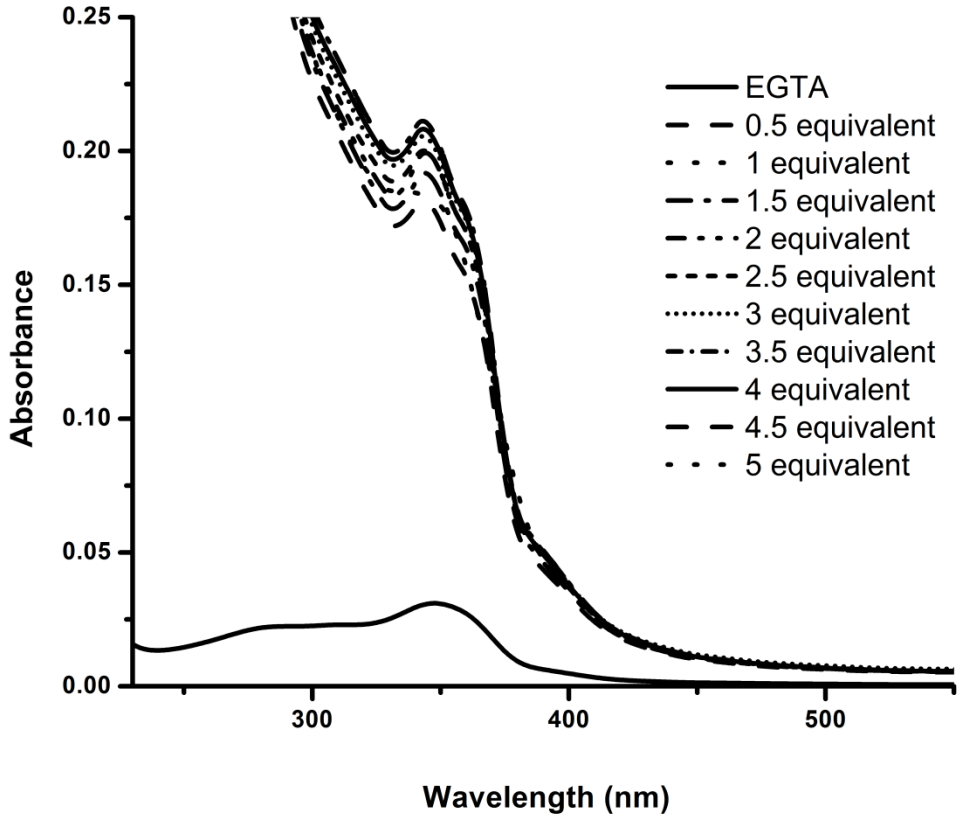


(A)

Supplementary Figure 3: 1D-NMR spectra of Ca/Na-1. (A)^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) Overlay of ^{23}Na spectra of Ca/Na-1 and NaOH standard solution. (D) Chemical structure of Ca/Na-1 showing peak labelling.



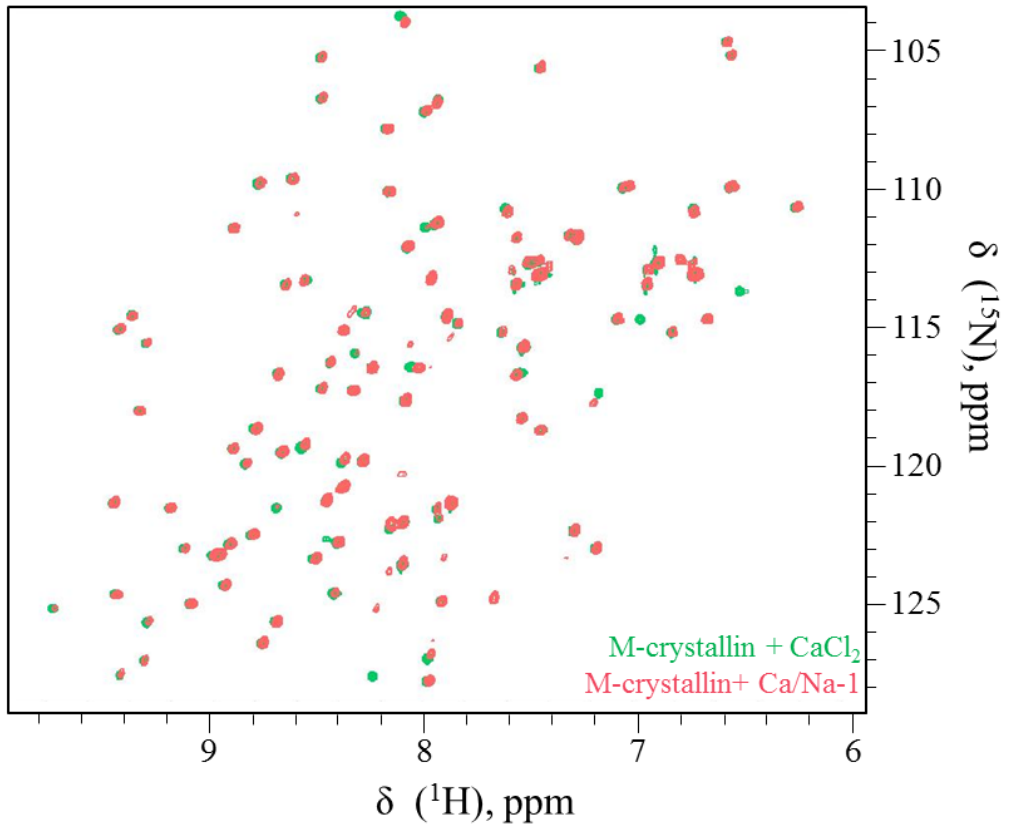
Supplementary Figure 4: UV-vis spectra of 10 mM EGTA solution (in presence of 4 equivalent NaOH) in presence of different equivalent of $Ca(NO_3)_2.4H_2O$.



Supplementary Table 3: Combined chemical shifts of 1D NMR spectra.

		Chemical Shifts (δ, ppm)	Coupling Constant (J, Hz)
^{1}H	H_1	3.8825 (s)	
	H_2	2.9346 (t)	
	H_3	3.467(d)	17.79
	H_4	3.7284 (s)	
	H_5	3.2816(d)	17.79
^{13}C	C_A	180.412	
	C_B	61.829	
	C_C	57.569	
	C_D	68.181 (d)	193.671
	C_E	69.144 (d)	193.671

Supplementary Figure 5: Comparison of M-crystallin titration using Ca/Na-1 and $CaCl_2$ as titrants. Spectral overlay of sensitivity enhanced [^{1}H-^{15}N] HSQC spectra of Ca^{2+} bound M-crystallin (green) and Ca/Na-1 bound protein (red). Spectra were recorded at 25 °C in 25 mM Tris-HCl buffer (pH 7.4) and 100 mM NaCl. (B) Chelation of Ca^{2+} at the two binding sites on M-crystallin. At each site, Backbone and side chain carbonyl 'O'atoms are involved in Ca^{2+} coordination (shown in green and blue respectively). The three coordinating water molecules are shown as yellow spheres.



Supplementary Table 4: **Thermodynamic parameters of M-crystallin-Ca/Na-1 complexation.** Isothermal titration calorimetry profile of the protein-Ca/Na-1 was carried out with protein concentration was 60 μM in 25 mM Tris-HCl buffer, pH 7.4 and 50 mM NaCl. 10 mM Ca/Na-1 stock solution was prepared in same buffer. The best fit values of macroscopic dissociation constants and ΔH obtained from isothermal titration calorimetry are listed.

Parameter	**Value**
K_1(M)	(1.9 ± 0.3) x 10^{-5}
ΔH_1 (kcal mol^{-1})	-13.4 ± 0.1
ΔS_1 [cal $(mol\ K)^{-1}$]	24.1
K_2 (M)	(1.4 ± 0.1) x 10^{-4}
ΔH_2 (kcal mol^{-1})	-5.9 ± 0.1
ΔS_2 [cal $(mol\ K)^{-1}$]	21.6

References

1. P. -C. Liang, H. –K. Liu, C. –T. Yeh, C. –H. Lin, V. Zima, *Cryst. Growth Des,* **2011**, *11*(3), 699-709.
2. G. M. Sheldrick, *Acta Crystallogr., Sect. A* 2008, **64**, 112−122.
3. A. L. Spek, *Acta Crystallogr., Sect. D* 2009, **65**, 148−155.
4. R. P. Barnwal, M. K. Jobby, K. M. Devi, Y. Sharma and K. V. R. Chary, *J Mol Biol.* 2009, **386**(3), 675–89.
5. R. P. Barnwal, M. K. Jobby, Y. Sharma and K. V. R. Chary, *J. Biomol. NMR,* 2006, **36**(1), 1-32.