Supplementary Figure-1: Sequential Ca$^{2+}$ filling pathway of myr NCS-1. The black spots represent Ca$^{2+}$ binding sites. Presence of Ca$^{2+}$ is represented by grey spot. A, B, C and D represents different species present in the equilibrium. A is apo protein, B represents protein where Ca$^{2+}$ is bound to site II, C represents protein where Ca$^{2+}$ is bound to sites II and III, and D represents protein where Ca$^{2+}$ is bound to sites II, III and IV. $k_{II}$ is the microscopic binding constant of Site II. $k_{III, II}$ is the microscopic binding constant of Site III when Site II is filled and so on.

A = [Apo-NCS-1]

B = [(Ca$^{2+}$)-(II)-NCS-1] (mono Ca$^{2+}$ occupied species where Ca$^{2+}$ is bound to site II)

C = [(Ca$^{2+}$)-(II)-(Ca$^{2+}$)-(III)-NCS-1] (species where Ca$^{2+}$ is bound to sites II and III)

D = [(Ca$^{2+}$)-(IV)-(Ca$^{2+}$)-(III)-(Ca$^{2+}$)-(II)-NCS-1] (Ca$^{2+}$ occupied species where Ca$^{2+}$ is bound to site II, III and IV)

$k_{II}$ = Microscopic Binding Constant of Site II.
k_{III, II} = \text{Microscopic Binding Constant of Site III when Site II is filled.}

k_{IV, II, III} = \text{Microscopic Binding Constant of Site IV when Sites III and II are filled.}

A + [Ca^{2+}] \rightarrow B \quad \text{(1)} \quad k_{II} = [B] / [A] [Ca^{2+}] \quad [B] = k_{II} [A] [Ca^{2+}]

B + [Ca^{2+}] \rightarrow C \quad \text{(2)} \quad k_{III, II} = [C] / [B] [Ca^{2+}] \quad [C] = k_{III, II} [A] [Ca^{2+}]^2

C + [Ca^{2+}] \rightarrow D \quad \text{(3)} \quad k_{IV, II, III} = [D] / [C] [Ca^{2+}] \quad [D] = k_{IV, II, III} [A] [Ca^{2+}]^3

A, B, C, D are equilibrium concentrations. [Ca^{2+}] \text{ is the free metal ion concentration at equilibrium.}

A + B + C + D = C_0 = \text{Total protein concentration} = 0.0008 \text{ M in this experiment} \quad \text{(4)}

Y = [Ca^{2+}] + B + 2C + 3D = \text{Total metal ion concentration}

Y = [Ca^{2+}] + k_{II}[A] [Ca^{2+}] + 2k_{II}k_{III, II}[A] [Ca^{2+}]^2 + 3k_{II}k_{III, II}k_{IV, III, II}[A] [Ca^{2+}]^3 \quad \text{(5)}

Taking [Ca^{2+}] = x , from (4) after expressing B, C, D in terms of A & x we get

\[ \frac{A}{C_0} = \frac{1}{1 + k_{II}x + k_{II}k_{III, II}x^2 + k_{II}k_{III, II}k_{IV, III, II}x^3} \quad \text{(6)} \]

Intensity of G78 peak in HSQC spectra was correlated to \((B+C+D)/C_0\) as in all the three species 2\text{nd} site is occupied with Ca^{2+}.

Now taking \(k_{II}k_{III, II}k_{IV, III, II} = a, \quad k_{II}k_{III, II} = b \text{ and } k_{II} = c\)

We define \(d = (B+C+D)/C_0\)

From eq (6) we can write \(d = (cx + bx^2 + ax^3)/(1 + cx + bx^2 + ax^3) \quad \text{(7)}\)

So from eq(7) we can write \(1 + cex + bex^2 + aex^3 = 0 \quad \text{--------(8)}\) where \(e = \{1-(1/d)\}\)

Defining \([Y/ C_0] = y, \text{ from equation (5) we get}\)

\[ y = \frac{x}{C_0} + \frac{3ax^3 + 2bx^2 + cx}{1 + cx + bx^2 + ax^3} \quad \text{(9)} \]

Eliminating x from equation (8) and (9) we can express \(y = f(e) \quad \text{--------(10)}\) keeping a, b, c as parameters. To obtain binding parameters (k_{II}, k_{III, II}, k_{IV, III, II}) a program called nllsq.f was used in
which for each e (experimentally observable) Equation 8 was solved for a given set of parameter values. Only real positive root was chosen and was then used in Equation 9. The fitting was performed following a least square fitting algorithm. The changes in the intensity of G78 peak in HSQC spectra were correlated to $(B+C+D)/C_0$ and the fitted curve is shown in supplementary Figure 2a. Nature of enormous complexity of Eq.(10) is the principal reason for the poor fitting in the initial phase of the reaction. We also have shown the normalized speciation graph (supplementary Figure 2b) of each individual species which support the sequential filling pathways.

Supplementary Figure-2: (A) Fitted plot of intensity profiles of the cross-peaks of G78 during the Ca$^{2+}$ titration. The blue line represents the simulated profile and the experimental curve is represented with (●). (B) Normalized speciation plot of different species present in the equilibrium. Green represents speciation plot of apo protein, red represents speciation plot of protein where Ca$^{2+}$ is bound to Site II, blue represents speciation plot of protein where Ca$^{2+}$ is bound to sites III and II, and grey represents speciation plot of protein where Ca$^{2+}$ is bound to site IV, III and II.