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Supporting Material

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SUPPLEMENTAL MATERIALS

Comparison of the thermodynamic landscapes of unfolding and formation of the energy dissipative state in the isolated light harvesting complex II

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Appendix 1A

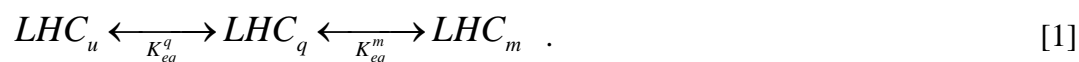
Thermodynamics of quenching and unfolding in LHC II

Theory. Upon incubation of LHC II (monomer or trimer) at different temperatures, two possible process need to be considered: the transition from the unquenched, folded, state of the complex to the quenched, folded, state and the transition either from unquenched or quenched form to an unfolded form of the protein. Previous investigations (*s1*, *s2*) suggested that, at least in LHC II trimers, the two transitions occur virtually independently, i.e. a complete transition to the quenched state is observed before any significant accumulation of the unfolded complex is detected. However, if transitions to the quenched and to the unfolded states of the complex are considered as *independent* it would appear that the quenched form of the complex is thermodynamically stable, irrespectively of the temperature. This scenario is obviously physically unsound. Therefore, a reaction scheme which considers *at least* one possible metastable intermediate needs to be taken into account.

In principle, several reaction mechanisms can describe overlapping temperature dependences between the transition to the quenched and unfolded form of the complex (Scheme 1). The conceptually simpler reaction scheme is defined by a linear three-stage model, in which the quenched state represents a meta-stable (folded) intermediate

between the unquenched (folded) form of the complex and the unfolded conformer(s) (Scheme 1A). A variation of this simple scheme involves considering off-pathway intermediates: in this scheme, unfolding and quenching occur from the native (unquenched) state of the complex and compete with each other (Scheme 1B). Both schemes require considering two equilibria only: the one between the unquenched and the quenched form of the complex and the other between the quenched and unfolded (Scheme 1A) or the unquenched and unfolded conformers (Scheme 1B). The most general case is a complete “triangular” reaction scheme (Scheme 1C), according to which protein denaturation occurs from both, the quenched and the unquenched states. It is difficult to distinguish *a priori* amongst these possibilities. However, at least in the trimeric form of LHC II, the transition to the quenched state has been reported to be nearly completed before a sizable amount of unfolded complexes is detected (refs. *s1*, *s2* and Figure 1). In this case, the transitions to the quenched form and to the unfolded form of the complex are almost sequential, making the off-pathway intermediate hypothesis less plausible. Though, we can not exclude the existence of parallel unfolding pathways from the quenched and unquenched folded forms of LHC II. However, the analysis of such reaction scheme would require considering three reaction equilibria, rather than two, which significantly increases the number of fit parameters and the uncertainty of the outcome.

Based on this rationale, the analysis of the thermodynamic properties of quenching and unfolding of LHC II was performed assuming a simple three-state process, involving two native, unquenched and quenched, states and the denatured one (this assumption is further discussed in the Results and Discussion). Extending the observations made for LHC II trimers to the monomers it is possible to write a following linear kinetic scheme:



At steady state, the concentration of each form of the complex is proportional to their corresponding molar fraction being in unquenched, quenched or denatured state. Provided that steady-state conditions are attained, at a given temperature, the respective

molar fractions can be expressed as a function of the quenching (K_{eq}^q) and melting (K_{eq}^m) equilibrium constants:

$$\gamma_u = \frac{1}{1 + K_{eq}^q + K_{eq}^q \cdot K_{eq}^m}; \gamma_q = \frac{K_{eq}^q}{1 + K_{eq}^q + K_{eq}^q \cdot K_{eq}^m}; \gamma_m = \frac{K_{eq}^q \cdot K_{eq}^m}{1 + K_{eq}^q + K_{eq}^q \cdot K_{eq}^m}, \quad [2]$$

The equilibrium constants K_{eq}^q and K_{eq}^m are defined by the mass action law:

$$K_{eq}^q = \frac{[LHC_q]}{[LHC_u]} = e^{-\frac{\Delta G_q^\circ(T)}{RT}}; K_{eq}^m = \frac{[LHC_m]}{[LHC_q]} = e^{-\frac{\Delta G_m^\circ(T)}{RT}}, \quad [3]$$

where R is the universal gas constant, T is the absolute temperature, $\Delta G_q^\circ(T)$ and $\Delta G_m^\circ(T)$ are the difference in the Gibbs free energies between the quenched and unquenched and the unfolded and quenched form of the complex, respectively, under standard conditions, i.e. equal molar fractions of products and reagents and 0.1 MPa (1 bar) atmosphere. The temperature dependence of $\Delta G_q^\circ(T)$ (and $\Delta G_m^\circ(T)$) with respect to a reference temperature T_r is described by the Gibbs-Helmholtz equation (Derivation is given in **Appendix 1B**):

$$\Delta G_q^\circ(T) = \Delta H_q^\circ(T_r) - T\Delta S_q^\circ(T_r) + \Delta C_{p,q}(T - T_r) - \Delta C_{p,q}(T \ln \frac{T}{T_r}), \quad [4]$$

where $\Delta H_q^\circ(T_r)$ and $\Delta S_q^\circ(T_r)$ are the standard quenching enthalpy and entropy differences at the reference temperature, respectively. $\Delta C_{p,q}$ is the difference in specific heat capacity between the *unquenched* (relaxed) and the *quenched* form of the LHC II complex. As previously mentioned, in general, the temperature dependence of $\Delta C_{p,q}$ is weak, so that it can be considered as a constant value within a certain temperature range. It is convenient to set the reference temperature to the value of the characteristic quenching temperature, T_q , which is defined as the temperature at which the molar fractions of the quenched and unquenched complexes are equal (at

equilibrium), so that $\Delta G_q^\ominus(T_q) = \Delta H_q^\ominus(T_q) - T_q \Delta S_q^\ominus(T_q) = 0$. Defining $\Delta H_q^\ominus \equiv \Delta H_q^\ominus(T_q)$ and $\Delta S_q^\ominus \equiv \Delta S_q^\ominus(T_q) = \frac{\Delta H_q^\ominus}{T_q}$, and further substituting these terms into Equation 4 when

$T_i = T_q$, the following expressions are obtained:

$$\begin{cases} \Delta G_q^\ominus(T) = \Delta H_q^\ominus \left(1 - \frac{T}{T_q}\right) + \Delta C_{p,q}(T - T_q) - \Delta C_{p,q} \left(T \ln \frac{T}{T_q}\right) \\ \Delta H_q^\ominus(T) = \Delta H_q^\ominus(T_q) + \Delta C_{p,q}(T - T_q) \\ T \cdot \Delta S_q^\ominus(T) = \Delta H_q^\ominus \frac{T}{T_q} + \Delta C_{p,q} \left(T \ln \frac{T}{T_q}\right) \end{cases} \quad [5]$$

Clearly an expression analogous to Equation 5 can be written for the transition to the unfolded state of the complex, upon definition of a characteristic melting temperature T_m .

Analysis of the activation barrier. The activation energy of an endergonic reaction, $\Delta G_q^*(T)$, can be expressed as the sum of $\Delta G_q^\ddagger(T)$, the free energies of activation of the reverse, exergonic reaction, and $\Delta G_q(T)$. The free energy of activation of the spontaneous reaction (the relaxation of quenching in this case) is also described by the Gibbs-Helmholtz equation. Expressing $\Delta G_q^\ddagger(T)$ for the same reference temperature T_q used to define $\Delta G_q(T)$ we obtain:

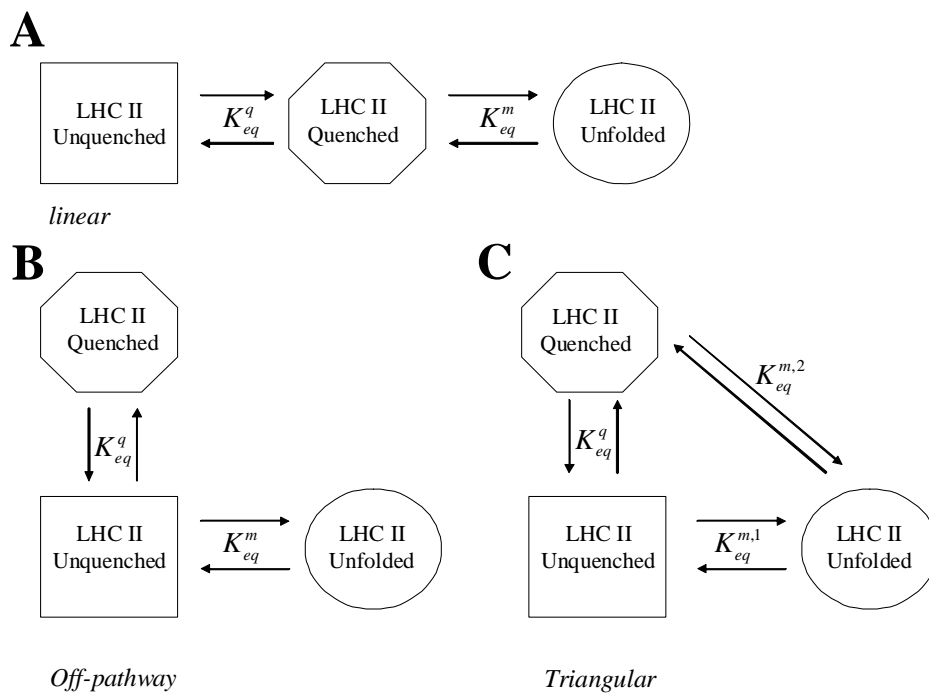
$$\Delta G_q^\ddagger(T) = \Delta H_q^\ddagger - T \Delta S_q^\ddagger + \Delta C_{p,q}^\ddagger (T - T_q) - \Delta C_{p,q}^\ddagger \left(T \ln \frac{T}{T_q}\right), \quad [6]$$

where ΔH_q^\ddagger is the activation enthalpy, $\Delta C_{p,q}^\ddagger$ is the differential heat capacity between the *quenched* form of the complex and the *transition* state and ΔS_q^\ddagger is the activation entropy. Similar treatments for the activation energy barrier have been discussed in protein folding and unfolding studies (*e.g.* **s3-s5**). It has been suggested that it is possible to describe discontinuous Arrhenius plots by simply taking into account the temperature dependence of $\Delta G_q(T)$ (**s6**). However, for the transition to quenched state of LHC II the omission of a differential heat capacity between the quenched and the transition state led to a less satisfactory fit of the experimental results.

The temperature dependencies of the quenching kinetics were fitted using the Eyring-Evans equation:

$$k(T) = \kappa \frac{k_b T}{h} \cdot e^{-\frac{\Delta G^*(T)}{RT}}, \quad [7]$$

where, κ is the transmission coefficient (i.e. the probability that the reaction takes place from the transition state), h is the Plank constant, k_b is the Boltzmann constant, and $\Delta G_q^*(T)$, is a linear combination of $\Delta G_q^\ddagger(T)$ (Equation 6) and $\Delta G_q^\ominus(T)$ (Equation 5).



Scheme 1. Possible kinetic models of the transition to the quenched and unfolded forms of LHC II. **A:** Linear three-stage reaction. **B:** Off-pathway reaction. **C:** Triangular reaction.

Appendix 1B.

Derivation of the Gibbs-Helmholtz Equation

Temperature dependence of the Enthalpy Difference, $\Delta H(T)$. Enthalpy is related to the heat capacity at constant pressure, C_p , by the relationship:

$$C_p = \frac{\partial H}{\partial T}. \quad [8]$$

For an infinitesimal increment in the surrounding of the temperature T_i it is possible to write, $dH = C_p dT_i$. Hence the enthalpy at a given temperature T can be described with respect of the temperature T_r as:

$$H(T) = H(T_r) + \int_{T_r}^T C_p dT. \quad [9]$$

Assuming that C_p is independent from temperature, which is generally true for small temperature differences, then the integral in equation [4] yield:

$$H(T) = H(T_r) + C_p(T - T_r). \quad [10]$$

Applying equation [4] for the transition between two states of the given system (unquenched/quenched, folded/unfolded, and so on) it is possible to calculate the enthalpy difference ΔH . As the case of interest is that of standard conditions, on then simply obtains:

$$\Delta H^\ominus(T) = \Delta H^\ominus(T_r) + \Delta C_p(T - T_r). \quad [11]$$

It is worth noticing that if ΔC_p , i.e. the difference in heat capacity between the final and the initial state, is zero then $\Delta H^\ominus(T)$ is temperature independent.

Temperature dependence of the Entropy Difference, $\Delta S(T)$. The derivation is analogous to those of $\Delta H^\ominus(T)$. The entropy of system in given state, at constant pressure, is related to the heat capacity by the following relation:

$$\frac{dS}{dT} = \frac{C_p}{T} . \quad [12]$$

The change in the entropy of the system, by changing the temperature from a reference T_r to an arbitrary temperature T , is then given, after separation of the variable, and in the assumption that C_p is temperature independent by:

$$\int_{T_r}^T dS = C_p \int_{T_r}^T \frac{dT}{T} . \quad [13]$$

From which:

$$S(T) = S(T_r) + C_p \ln \frac{T}{T_r} . \quad [14]$$

Again, by the taking the difference between two states of the system and considering standard conditions, it is possible to write:

$$\Delta S^\ominus(T) = \Delta S^\ominus(T_r) + \Delta C_p \ln \frac{T}{T_r} \quad [15]$$

Substituting Equation [11] and Equation [15] into the definition of Gibbs free energy difference $\Delta G^\ominus(T) = \Delta H^\ominus(T) - T\Delta S^\ominus(T)$ yields:

$$\Delta G^\ominus(T) = \Delta H^\ominus(T_r) - T\Delta S^\ominus(T_r) + \Delta C_p(T - T_r) - \Delta C_p(T \ln \frac{T}{T_r}) . \quad [16]$$

Appendix 2

Fitting procedure

The circular dichroism signal at a given wavelength and a given temperature can be expressed as:

$$CD(\lambda, T) = [LHC]_0 (\gamma_u(T) \cdot \Delta\varepsilon_u(\lambda) + \gamma_q(T) \cdot \Delta\varepsilon_q(\lambda) + \gamma_m(T) \cdot \Delta\varepsilon_m(\lambda)) = [LHC]_0 (\gamma_q(T) \cdot \Delta\Delta\varepsilon_{uq}(\lambda) + \gamma_m(T) \cdot \Delta\Delta\varepsilon_{um}(\lambda)) \quad , \quad [1]$$

where $[LHC]_0$ is the initial concentration (in moles) of the sample (which is known and it is a constant), $\Delta\varepsilon_u(\lambda)$, $\Delta\varepsilon_q(\lambda)$ and $\Delta\varepsilon_m(\lambda)$ are the molar ellipticity of the unquenched, quenched and unfolded form of LHC II, respectively. The double differential extinction coefficient are defined as $\Delta\Delta\varepsilon_{uq}(\lambda) = \Delta\varepsilon_u(\lambda) - \Delta\varepsilon_q(\lambda)$ and $\Delta\Delta\varepsilon_{um}(\lambda) = \Delta\varepsilon_u(\lambda) - \Delta\varepsilon_m(\lambda)$. The molar fractions of quenched and unquenched complexes, as a function of the temperature, are determined by the equilibrium constant, K_{eq}^q (Equation 2, Supplementary Material Appendix 1) and hence by the Gibbs free energy, $\Delta G_q^\ominus(T)$ (Equation [11], **Appendix 1**). The thermodynamic quantities are retrieved from fitting the experimental results to a model function in which $\Delta\Delta\varepsilon_{uq/um}(\lambda)$, $\Delta H_{q/m}^\ominus$, $\Delta C_{p,q/m}$ and $T_{q/m}$ are free running parameters by a non-linear least square routine, which minimises the reduced sum of χ^2 . The errors associated with each data-point were estimated by standard deviation of 5-8 independent measurements and therefore describe inter-sample variability.

The best-fit values of $\Delta H_{q/m}^\ominus(T)$ and $\Delta C_{p,q/m}$ display partial cross correlation, based on covariance matrix analysis, as both contribute to determining the value of $\Delta G_{q/m}^\ominus(T)$ and $K_{eq}^{q/m}$. Thus, in order to reduce the number of possible solutions and to increase the accuracy in estimation of the model parameters, the temperature dependence of the activation energies and steady-state equilibria were simultaneously fitted (global analysis). Moreover, in order to reduce the impact of initial guesses on the fit estimate, the minimisation has been conducted by using initially the more robust simplex algorithm followed by the more accurate, but more prone to entry-guess-biases, Levenberg-Marquardt algorithm.

Estimation of the confidence on the best fit parameters. The stability of the fit solutions was tested as described by Beechem (**s7**). To evaluate the error associated

with each parameter, the value of a best-fit estimate was altered systematically over a certain range, and a series of non-linear least square minimisation was performed from this new initial condition. The same procedure was repeated iteratively for the all studied parameters. The distribution of values obtained for each parameter, as a result of the iterative perturbation, was then compared to the uncertainties calculated using the covariance matrix method, within the 2σ confidence level. Typically, a good agreement was observed between the different methods used to estimate the errors associated with the best-fit. In general, the errors associated with $\Delta H_{q/m}^{\circ}(T)$ and $T_{q/m}$ were small, i.e. within 5-15% and 1-5% of the best-fit value. Somewhat larger errors were associated with $\Delta C_{p,q}$, up to 25-30% of the best-fit estimate, largely due to the small absolute value of $\Delta C_{p,q}$ is small. In order to show the range of possible acceptable solutions the levels of confidence associated with the fits are presented.

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