Supporting Information to:

Molecular organization of cytochrome c_2 near the binding domain of cytochrome bc_1 studied by electron spin-lattice relaxation enhancement.

Rafał Pietras, Marcin Sarewicz, Artur Osyczka

Department of Molecular Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology,

Jagiellonian University, Kraków Poland

Analysis of Inversion Recovery (IR) curves

All relaxation times were determined from analysis of Inversion Recovery curves. As this approach is often reported to be influenced by spectral diffusion we tested if this process can significantly contribute to our recovery curves. Fig. S1, *A* compares the results obtained from IR with those registered with the use of a saturating picket fence sequence of ten π pulses. We found no differences between curves obtained with the IR and the picket fence sequence therefore we concluded that under our conditions the spectral diffusion has no significant contribution to the registered recovery curves. Irrespective of the applied pulse sequence the registered recovery curves were non-single exponential.

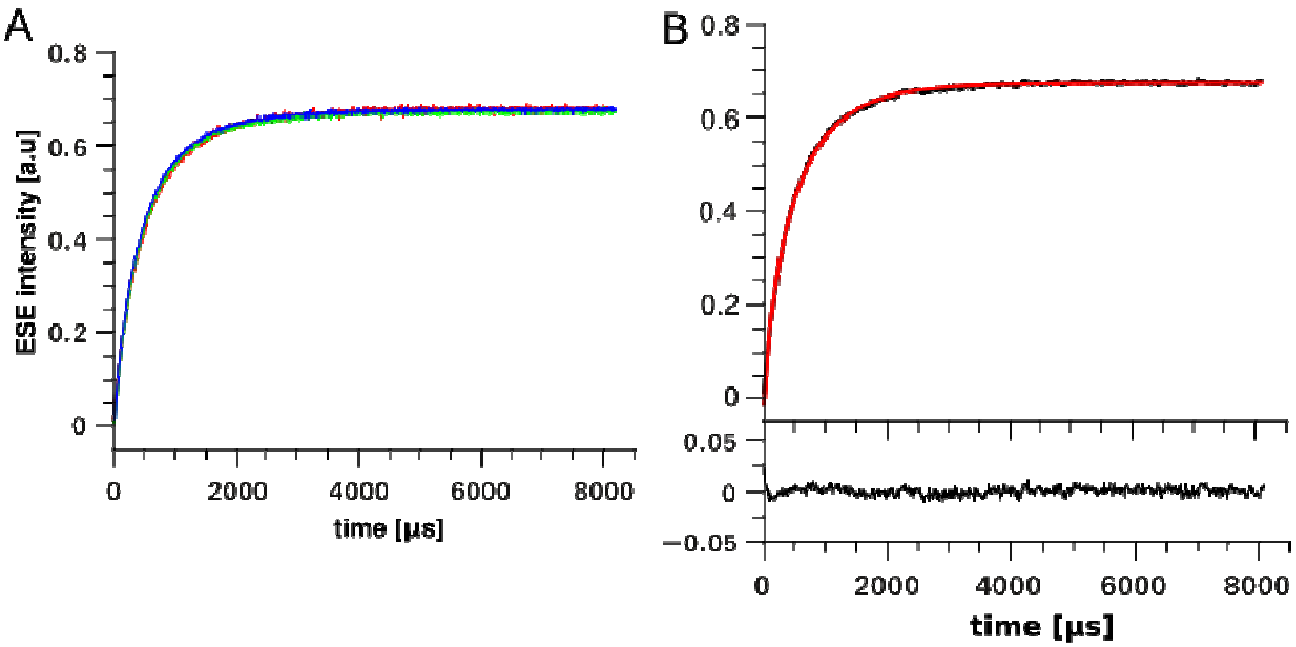

Figure S1. **Raw experimental recovery curves obtained for A101C-SL at 95K**. *A* – comparison of recovery curves obtained with the use of an IR sequence (black) with those obtained with the use of a saturating picket fence sequence of ten 40 ns π-pulses. Several spacing times between pulses in the picket fence sequence were tested: 100 ns (red), 1 µs (green), 10 µs (blue). *B* – IR curve fitted with stretched exponent. Obtained parameters were $\tau = 491 \pm 3$ µs and $\beta = 0.807 \pm 0.004$

The recorded IR curves were normalized, multiplied by -1 and offset correction was applied. The curves were fitted with the stretched exponent function:

$$I(t) = \exp\left(-(t/\tau)^{\beta}\right) \tag{1}$$

This function describes a distribution of spin-lattice relaxation times[1], where $\beta \in (0,1)$ and is related to the width of the distribution, and τ is the time constant (see Fig. S1, *B* for an example of fitting). The area under the curve can be interpreted as an average relaxation time and can by calculated as follows:

$$\tau_{avr} = \frac{\tau}{\beta}\Gamma\left(\frac{1}{\beta}\right) \tag{2}$$

where: Γ is the Euler gamma function.

The average spin-lattice relaxation rate is then equal to:

$$k_1 = \frac{1}{\tau_{avr}} \tag{3}$$

In all cases the extent of paramagnetic relaxation enhancement (PRE) refers to a difference in the relaxation rate for sample with PRE present (k_1^{PRE+}) and that in which the PRE was absent (k_1^{PRE-}):

$$k_{dip} = k_1^{PRE+} - k_1^{PRE-} \tag{4}$$

Obtaining the binding parameters from IR measurements

To analyze the binding of cytochrome c_2 to cytochrome bc_1 we assumed that the process is described by a two-state model in which A101C-SL experiences different PRE in bound (to cytochrome bc_1) and unbound forms. It follows that the measured IR curve can be represented as a sum of two components:

$$I(t) = f_b \exp\left(-\left(t/\tau_1\right)^{\beta_1}\right) + f_{ub} \exp\left(-\left(t/\tau_2\right)^{\beta_2}\right) \tag{5}$$

where: f_b is the fraction of bound A101C-SL molecules, τ_1 and β_1 are the parameters of the stretched exponent in the bound state, f_{ub} is the fraction of unbound A101C-SL molecules, τ_2 and β_2 are the parameters of stretched exponent of the unbound state.

Using the expression (2), one is able to calculate the area under the IR curve. This gives the representation of IR curves (Eq. 5) in terms of average spin-lattice relaxation time:

$$\tau_{avr} = f_b \tau_{avr}^{b} + f_{ub} \tau_{avr}^{ub} \tag{6}$$

where: τ_{avr}^{b} , τ_{avr}^{ub} are the average relaxation times of A101C-SL in the bound and unbound states, respectively.

As $f_b = 1 - f_{ub}$, the equation (6) can be rewritten to describe τ_{avr} as a function of the bound fraction of cytochrome c_2:

$$\tau_{avr} = f_b\left(\tau_{avr}^{b} - \tau_{avr}^{ub}\right) + \tau_{avr}^{ub} \tag{7}$$

According to Eq. (7), the average relaxation time is a linear function of bound cytochrome c_2. Thus, the

analysis of the titration of A101C-SL with cytochrome bc_1 was performed with the use of the relaxation times instead of relaxation rates.

The bound fraction (f_b) of cytochrome c_2 can be expressed as a function of the total cytochrome bc_1 concentration (P)[2,3]:

$$f_b(P) = \frac{1}{2L_0}\left(K_d + L_0 + n_a P - \left((K_d + L_0 + n_a P)^2 - 4 n_a P L_0 \right)^{\frac{1}{2}} \right) \quad (8)$$

where: L_0 is the total cytochrome c_2 concentration, K_d is the dissociation constant, n_a is the number of binding sites. Fitting of Eq. (7) and Eq. (8) to our data, with n_a treated as a parameter, failed to reproduce the experimental binding curves in the case of low ionic strength conditions (Fig. S2). Thus we proposed a slight modification of Eq. (8) by the replacing n_a parameter with the following function:

$$n_a = \alpha \exp(-P/L_0) + n \quad (9)$$

In the limit of $L_0 >> P$ (i.e. for initial points of titration) the n_a reaches the value of $\alpha + n$. This should be considered as a process in which many cytochrome c_2 molecules are gathered near the binding domains. As the concentration of P grows, the n_a value gradually decreases reaching n under conditions where $P >> L_0$. This reflects the process of dispersion of cytochrome c_2 molecules to occupy the larger population of available binding sites. The comparison of the fitting of Eq. 8 before and after the modification is shown in figure S2.

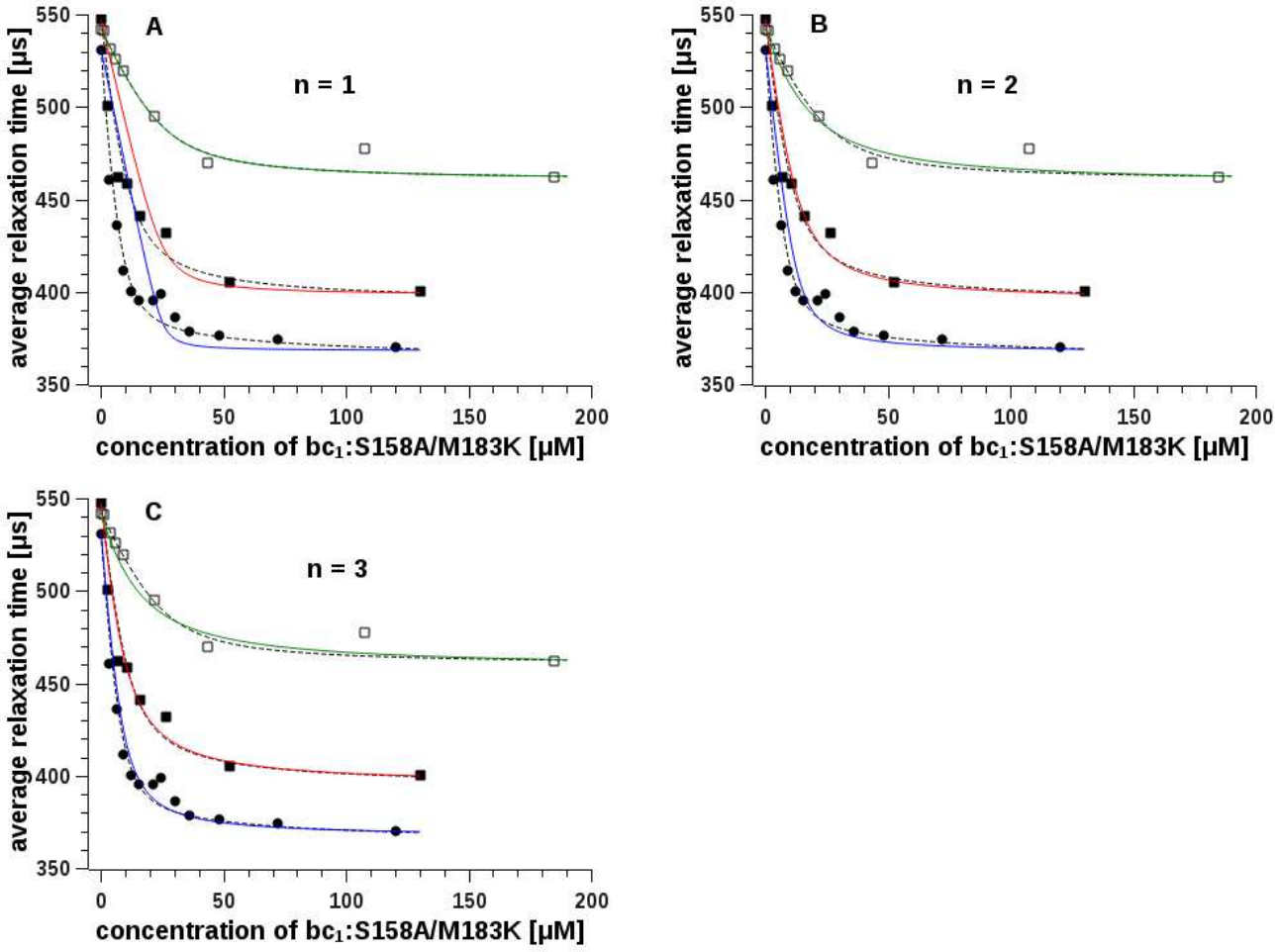

Figure S2. **Comparison of models describing the cytochrome c_2 binding isotherms**.

A) Results of fitting the data with model assuming n = 1. The K_d values are: 0.11, 0.6 and 5.1 µM for 0, 10 and 25 mM NaCl respectively. B) The same as in A but using model assuming n = 2. The K_d values are: 2.7, 3.2 and 20 µM for 0, 10 and 25 mM NaCl respectively. C) The same as in A but using model assuming n = 3. The K_d values are: 6, 12, 32 µM for 0, 10 and 25 mM NaCl respectively. The dotted lines shows fits based on the model which assumes that number of cytochrome c_2 molecules localized near the binding domain of cytochrome bc_1 changes with the ionic strength and the cytochrome bc_1 : cytochrome c_2 molar ratio. (see Eq. 1 and Eq. 2 in the main text).

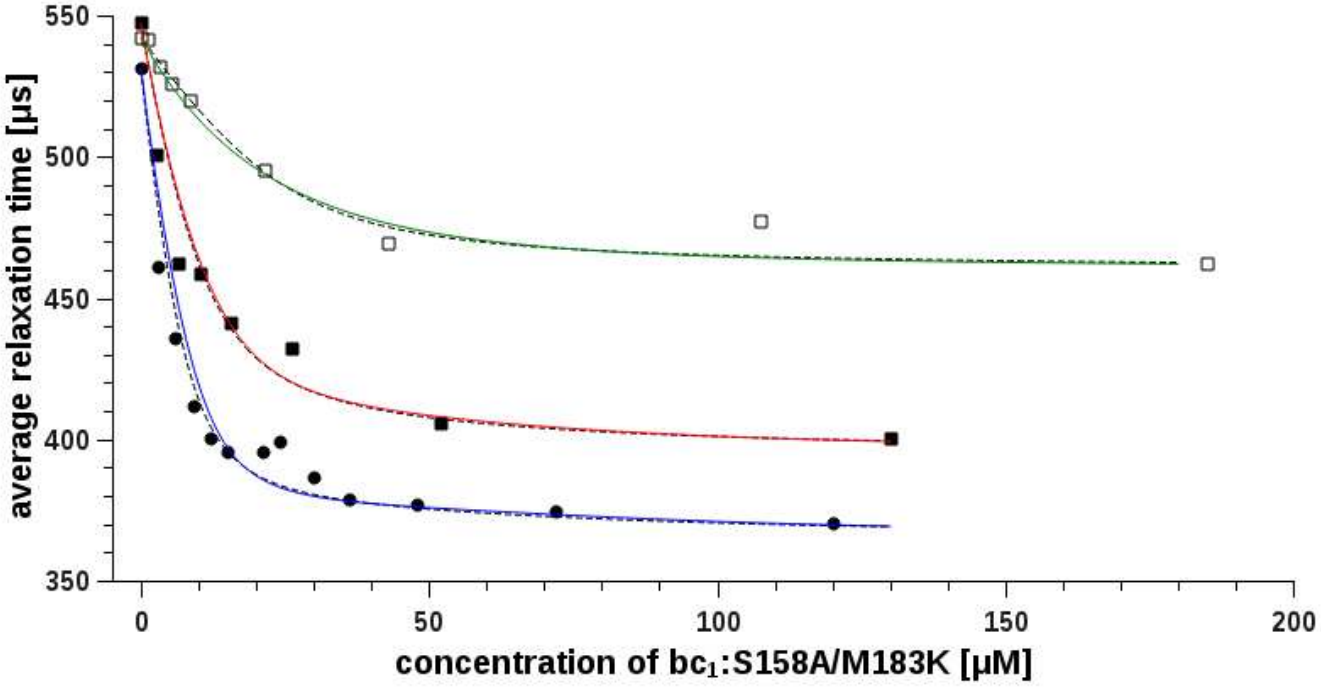

Figure S3. **Correlation between binding stoichiometry and dissociation constant** (K_d). The data points are marked as in Fig. S2. The curves were fitted assuming two models: first with n = 1 (one molecule of cytochrome c_2 binds to cytochrome bc_1 monomer) (black dashed lines) and the second with n = 0.5 (one molecule of cytochrome c_2 binds to cytochrome bc_1 dimer) (colored lines). Changes in the binding stoichiometry at the same time are compensated by changes in K_d (Table S1). In both cases the fitted curves are virtually indistinguishable.

	$n = 1$		$n = 0.5$	
NaCl [mM]	K_d [μM]	α	K_d [μM]	α
0	2.7	2.1	1.1	2.1
10	3.9	1.2	1.2	1.5
25	5.1	0	1.7	0.7

Table S1. **Comparison of binding parameters** obtained from fitting the titration data with the assumption that binding stoichiometry is one cytochrome c_2 per cytochrome bc_1 monomer ($n = 1$) and one cytochrome c_2 per cytochrome bc_1 dimer ($n = 0.5$). For each titration experiment one can obtain two different sets of parameters that reproduce the data equally well.

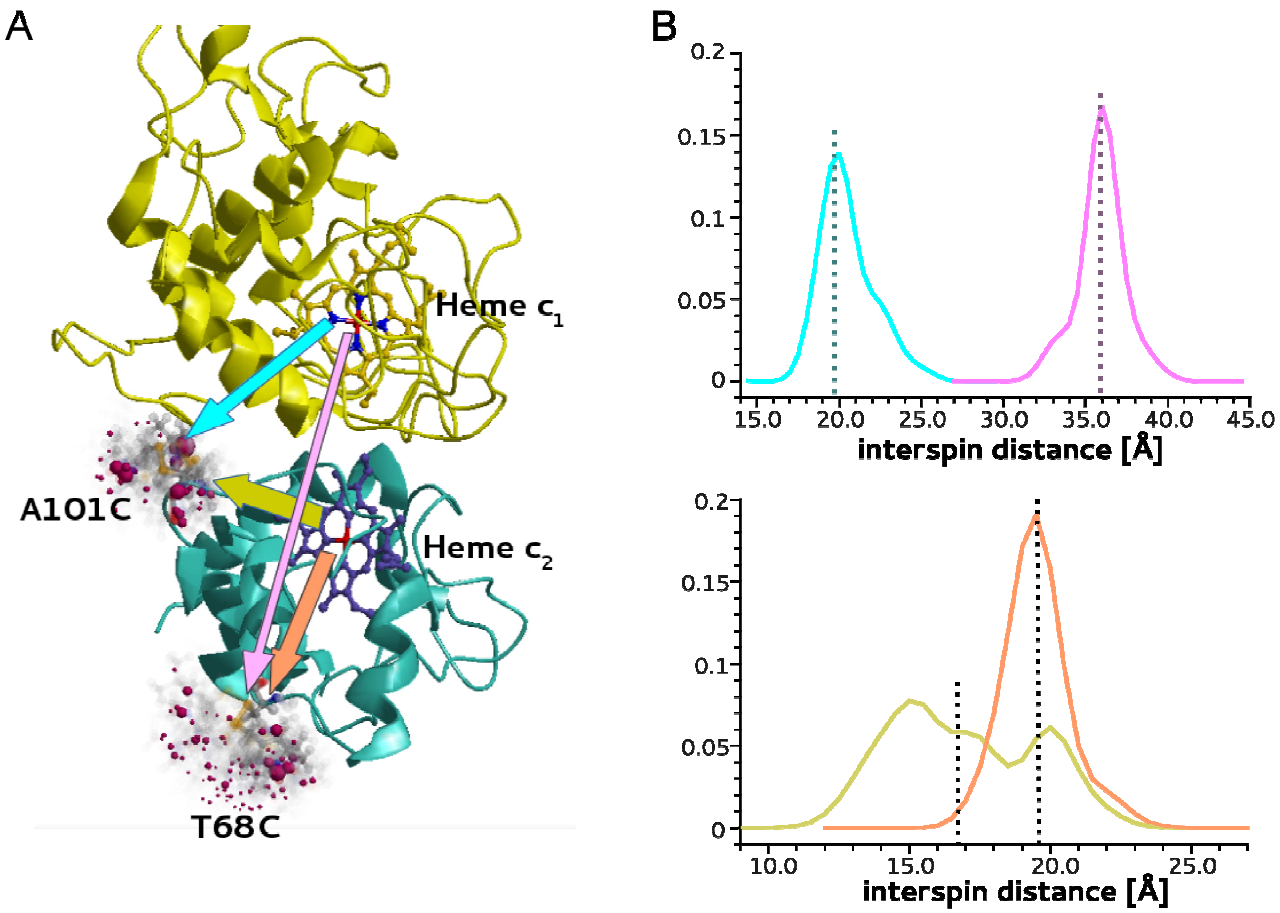

Figure S4. **Visualization of SL rotamers** on the model structure of cytochrome c_2 (cyan) bound to cytochrome c_1 (yellow) subunit of cytochrome bc_1. The structures of rotamers at each labeling site were obtained in MMM software with the use of MTSL rotamer library (A). Violet spheres indicate the position of NO group and their size is proportional to population. The arrows indicate four possible instances of PRE that can be observed in this system separately. The thickness of the arrows represents the strength of PRE. Distance distribution between NO groups and the iron atom of a given heme was calculated from obtained rotamer structures (B). The colors on the plots are consistent with the colors of the arrows.

Construction of dipolar ruler

The dipolar ruler curve was constructed on the basis of Bloembergen theory[4]. The original formula describes the dipolar relaxation rate of a slowly relaxing center caused by a fast relaxing center. However, the exact calculation of k_{dip} can be made for a rigid system in which a distance between centers and angles between the principal g-tensor axes of interacting spins are fixed[5–9]. It is also needed to know the spectra, spin-spin and spin-lattice relaxation times for each interacting center. These conditions are not met in our system containing SL attached to cytochrome c_2 and heme c_1 in cytochrome bc_1. We were unable to determine the relaxation rate of the heme c_1 iron, because its CW EPR spectrum is barely measurable, as all its transitions are overlapped by much stronger signals coming from other metal centers of cytochrome bc_1, mainly heme b_H, and the Rieske iron-sulfur cluster. In our analysis we followed the simplifications of the Bloembergen equation described in[10], reaching the point where k_{dip} is approximated with a simple function:

$$k_{dip}(r) = a*r^{-6} \quad (10)$$

where: r is a distance and a is a scaling factor. The a value, that includes all non-distance-related factors of the Bloembergen equation, was determined from fitting the Eq. (10) to the points for which distances were known and k_{dip} were measured. Such an approach, similar to that previously applied[11], is valid only when we assume that paramagnetic properties of heme c_2 and heme c_1 are comparable. Additionally we assume that for all samples the effect of changes in the relative orientation between g-tensors of SL and hemes has a negligible impact on k_{dip}[12] in comparison to the effect of changes in the distance.

References

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