

APPENDIX 1

Calculation of the shifted heme spectra.

The sum of the first three b -spectra, b_{123} , contains spectral contributions from electron back-flow, ΔA_e , and heme a_3 relaxations, ΔA_r , the former being the major component:

$$b_{123} = \Delta A_e + \Delta A_r$$

The model (m) spectrum for the electron back-flow, ΔA_m , is:

$$\Delta A_m = (a_{3m}^{2+} - a_{3m}^{3+}) + (a_m^{3+} - a_m^{2+})$$

where the two difference spectra in the parentheses are obtained from bench-made spectra in the following manner:

$$(a_{3m}^{2+} - a_{3m}^{3+}) = (\text{FR} - \text{FRCO}) - (\text{Ox} - \text{MVCO})$$

$$(a_m^{3+} - a_m^{2+}) = (\text{MVCO} - \text{FRCO}) - \text{Cu}_A$$

where Cu_A is the published spectrum of Cu_A^{2+} (23); the absorbance of the reduced form is assumed to be negligible. The contribution from the heme a_3 relaxation is:

$$\Delta A_r = a_{3m}^{*2+} - a_{3m}^{2+}$$

where a_{3m}^{*2+} refers to the unrelaxed state of heme a_3^{2+} . Subsequently ΔA_m and ΔA_r are combined to create the unrelaxed model spectrum, ΔA_m^* :

$$\Delta A_m^* = (a_{3m}^{2+} - a_{3m}^{3+}) + (a_m^{3+} - a_m^{2+}) + (a_{3m}^{*2+} - a_{3m}^{2+}) = (a_{3m}^{*2+} - a_{3m}^{3+}) + (a_m^{3+} - a_m^{2+})$$

The modified first term can be rewritten as a difference between two difference spectra:

$$(a_{3m}^{*2+} - a_{3m}^{3+}) = (a_{3m}^{*2+} - a_{3m}^{2+} \text{ CO}) - (a_{3m}^{3+} - a_{3m}^{2+} \text{ CO})$$

Note that the first difference spectrum is the spectrum of the first intermediate in our MV flash experiment, practically the unrelaxed 100 ns spectrum, In1. The second difference spectrum is Ox minus MVCO. Thus the unrelaxed model spectrum for the electron back-flow can be obtained from the experiment and the bench-made model spectra:

$$\Delta A_m^* = \ln 1 - (\text{Ox} - \text{MVCO}) + (\text{MVCO} - \text{FRCO}) - \text{Cu}_A$$

Using the unrelaxed model spectrum simplifies our task because we can now compare b_{123} with ΔA_m^* more precisely since both of them have the relaxation contribution, and consequently a more reasonable value can be obtained for f , the fraction of the electron back-flow. From this comparison, we estimate f to be 0.75, and from the discrepancies between the experimental and model spectra we conclude that the actual model for the back-flow should contain shifted spectra for heme a_3^{3+} and heme a^{2+} , the oxidation states formed as a result of the electron back-flow. Thus the experimental b_{123} should be written as:

$$b_{123} = (\Delta A_m^* + \Delta A_s) \times f + (1 - f) \times \Delta A_r$$

where $\Delta A_s = (a_{3m}^{3+} - a_{3s}^{3+}) + (a_m^{2+} - a_s^{2+})$ represent the differences between the unshifted and the shifted (s) absolute spectra of heme a_3^{3+} and heme a^{2+} . The term $(1 - f) \times \Delta A_r$ is the fraction of the relaxation contribution unaccounted for due to f being less than 1. It is not significant because the fraction of the back-flow is high, 0.75, and the spectral contribution from the relaxation itself is not large. Therefore, we will ignore the relaxation term in calculation of the spectral shifts:

$$\Delta A_s \times f = b_{123} - \Delta A_m^* \times f$$

The double difference spectrum in Figure 5b (solid curve) was constructed according to this equation, and it shows the superposition of two difference spectra presumed to originate from the shifted heme spectra. Each difference spectrum can be simulated by shifting Gaussians along the wavelength axis and taking the difference between the unshifted and shifted Gaussians, Δg_1 and Δg_2 . The Gaussians were selected based on the

positions and widths of the published absolute spectra of heme a_3^{3+} and heme a^{2+} (45).

The sum of the two simulated spectra is shown in Figure 5b (dashed curve)

$$\Delta A_s \times f = \Delta g_1 + \Delta g_2$$

where the first term is assigned to the heme a_3^{3+} shift and the second term to the heme a^{2+} shift:

$$\Delta g_1 = (a_{3m}^{3+} - a_{3s}^{3+}) \text{ and } \Delta g_2 = (a_m^{2+} - a_s^{2+})$$

Using the simulated Gaussian difference spectra and the unshifted model spectra, we can calculate the shifted oxidized-minus-reduced spectrum of heme a_3 and reduced-minus-oxidized spectrum of heme a , made up of the shifted absolute spectra of heme a_{3s}^{3+} or heme a_s^{2+} . The shifted difference spectra:

$$(a_{3s}^{3+} - a_{3m}^{2+}) = (a_{3m}^{3+} - a_{3m}^{2+}) + \Delta g_1 / f$$

$$(a_s^{2+} - a_m^{3+}) = (a_m^{2+} - a_m^{3+}) - \Delta g_2 / f$$

are shown in Figure 6.