**Supplementary information:**

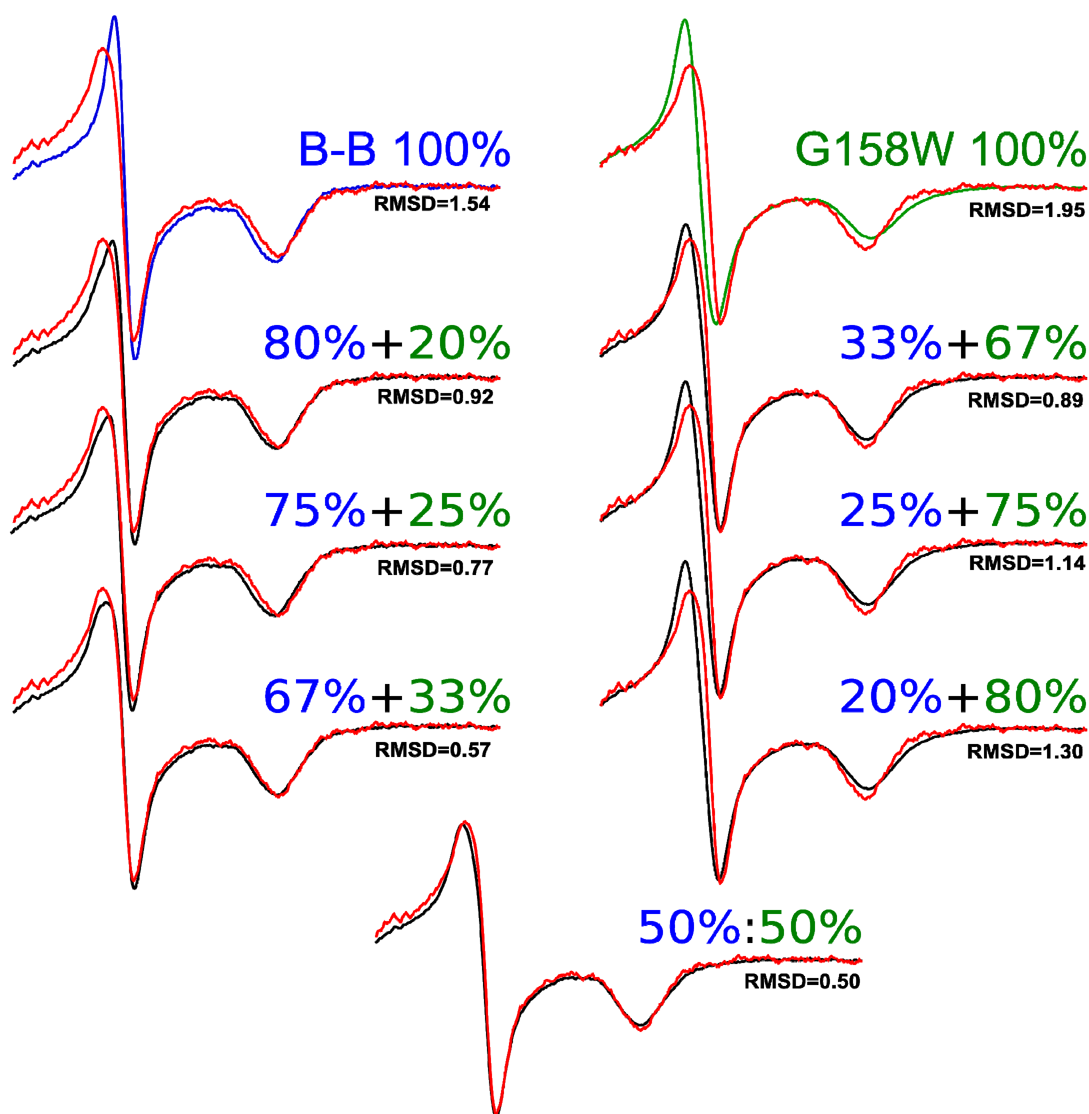


**Fig. S1**. Analysis of the spectral components in the CW EPR spectrum of the FeS cluster of asymmetric $_W$B-B protein. The spectrum measured for isolated asymmetric protein (red) was compared with the series of „synthetic" spectra (black) that were obtained by addition of the reference spectra in different proportions. As the reference we chose two spectra of the FeS cluster measured for B-B protein (non-disabled $Q_o$ sites) (blue) and G158W mutant (both $Q_o$ sites disabled) (green). Those reference spectra were first normalized against the amplitude of $g_y$ transition and subsequently added together in different proportions indicated in the figure. Each „synthetic" spectrum was subsequently compared with the experimental spectrum of $_W$B-B protein by calculating the RMSD values over the entire spectral range shown in the figure. The lowest RMSD (the smallest differences in the shape of the compared spectra) was obtained when spectra for B-B and G158W mutant were added in equal proportions (bottom). This means that in the asymmetric protein, the EPR spectrum reflects the presence of two equally numerous populations of FeS clusters: first interacting with the non-disabled $Q_o$ site and the second interacting with the disabled $Q_o$ site. It should be noted that all spectra were measured at exactly same conditions and were not processed afterward except for the normalization of the amplitudes of B-B and G158W to approximate equal spin concentrations.

**Considerations regarding the $V_{max}$ of $_{W}$B-B$^{N}$:**

Using the formula shown below we considered what percentage of highly active form would have to contaminate the isolated preparations of $_{W}$B-B$^{N}$ to produce the observed enzymatic activities provided that $_{W}$B-B$^{N}$ itself is as active as mixture of symmetrically inactivated mutants G158W and H212N in equal proportions.

If the calculations are preformed using the B-B level of enzymatic activity as the reference level for the highly active "contaminants", then the calculated percentage of contamination would have to be 14 %.

However, for the fused gene to loose both mutations to revert to pseudo-native form (and maintain the length of the gene) requires a two-step recombination process, which is expected to be very rare. In this respect, more probable would be a single-step recombination which amongst other possibilities could yield asymmetric forms with only one half of the fusion protein reverted to original sequence. If the level of those forms is taken as the reference level for the highly active "contaminants", then the calculated percentage of contamination increases to 23 % (taking $V_{max}$ of $_{W}$B-B).

We note that the two plasmid system with two tags (*1, 2*), the only currently available alternative for introducing asymmetric mutations to this protein, faces similar recombination possibilities. However in this case, the recombinations are far more difficult to monitor. While in the fusion protein system, at least the presence of the fused gene and its sequence can be monitored to verify that the predominant majority of enzyme would contain desired mutations, in the two tags system those options are not available (the presence of two tags in some fractions of isolated protein does not guarantee that recombinations between homologous portions of the genes not containing tags did not take place).

*Details of the calculations*

Assuming that the activity of $_{W}$B-B$^{N}$ is equal to the sum of 0.5 $V_{max}$ of G158W form ($V^{W}_{max}$ = 5 s$^{-1}$) and 0.5 of $V_{max}$ of H212N form ($V^{N}_{max}$ = 21 s$^{-1}$), the contribution to the total measured $V_{max}$ of asymmetric protein is decribed as:

$$V_{WB\text{-}BN} = 0.5\, f\, (V^{W}_{max} + V^{N}_{max}) = 13f, \qquad \text{(Eq S1)}$$

where $f$ is the fraction of $_{W}$B-B$^{N}$ protein in the solution.

If the fraction of contaminant protein (1-$f$) is characterized by the highest measured activity equal to $V_{max}$ of B-B form (408 s$^{-1}$), solving the equation:

$$V_{m} = (1-f)\, V_{C} + V_{WB\text{-}BN} \qquad \text{(Eq S2)}$$

where $V_{m}$ is the measured $V_{max}$ and $V_{C}$ is $V_{max}$ of contaminant protein;
gives $f = 0.86$ which means that the maximum fraction of the highest active contaminant protein is 14 %. Similar calculation performed assuming that $V_{C}$ is equal to $V_{max}$ of $_{W}$B-B (267 s$^{-1}$) gives $f$ = 0.77 (a contaminant fraction is at 23%).

References:


1. Castellani, M., Covian, R., Kleinschroth, T., Anderka, O., Ludwig, B., and Trumpower, B. L. (2010) Direct demonstration of half-of the sites reactivity in the dimeric cytochrome $bc_1$ complex, *J. Biol. Chem. 285*, 502-510.
2. Lanciano, P., Lee, D.-W., Yang, H., Darrouzet, E., and Daldal, F. (2011) Intermonomer electron transfer between the low-potential hemes of cytochrome $bc_1$, *Biochemistry 50*, 1651-1663.