Supporting Text

The thermodynamic analysis of a triple mutant shown below is a special case of the general theory of mutagenesis advanced by Horowitz and Fersht (1). Fig. 9 shows that the net free energy effect of a triple mutant (1, 2, 3) must be the sum of any path of mutations that leads from wild-type to the triple mutant. For the path highlighted in red:

$$\Delta G_{1,2,3} = \Delta G_1 + \Delta G_{2|1} + \Delta G_{3|1,2}, \qquad \textbf{[1]}$$

where $\Delta G_{2|1}$ is the energetic effect of mutation 2 in the background of mutation 1, and $\Delta G_{3|1,2}$ is the energetic effect of mutation 3 in the background of the double mutation at sites 1 and 2. To simplify Eq. **1**, we use the linkage relationships

(**a**) $\Delta G_{2|1} = \Delta G_2 - \Delta\Delta G_{1,2}$, and

(**b**) $\Delta G_{3|1,2} = \Delta G_{3|1} - \Delta\Delta G_{2,3|1}$

where $\Delta\Delta G_{2,3|1}$ is the two-way coupling free energy of mutations 2 and 3 in the background of mutation 1. The component terms of equation **b** can be further simplified by applying the following relationships:

(**c**) $\Delta G_{3|1} = \Delta G_3 - \Delta\Delta G_{1,3}$

(**d**) $\Delta^3 G_{1,2,3} = \Delta\Delta G_{2,3} - \Delta\Delta G_{2,3|1}$

Substituting equations **a**-**d** into Eq **1** and collecting terms, we find that

$\Delta G_{1,2,3} = \Delta G_1 + \Delta G_2 + \Delta G_3 - \left(\Delta\Delta G_{1,2} + \Delta\Delta G_{1,3} + \Delta\Delta G_{2,3}\right) + \Delta^3 G_{1,2,3}$, a relationship identical to Eq. **2** in the text. Thus, the free energy change of a triple mutant is the summed effect of the single mutant effects, the two-way thermodynamic couplings between them, and the three-way coupling energy.

1. Horovitz, A. & Fersht, A. R. (1990) *J. Mol. Biol.* **214,** 613-617.