Double-mutant cycles: a powerful tool for analyzing protein structure and function

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A double-mutant cycle involves wild-type protein, two single mutants and the corresponding double mutant protein. If the change in free energy associated with a structural or functional property of the protein upon a double mutation differs from the sum of changes in free energy due to the single mutations, then the residues at the two positions are coupled. Such coupling reflects either direct or indirect interactions between these residues. Double-mutant cycle analysis can be used to measure the strength of intramolecular and intermolecular pairwise interactions in proteins or protein–ligand complexes with known structure. Double-mutant cycles can also be employed to characterize structures that are inaccessible to NMR and X-ray crystallography, such as those of transition states for protein folding, ligand binding and enzyme catalysis, or of membrane proteins. Multidimensional mutant cycle analysis can be used to measure higher-order cooperativity between intramolecular or intermolecular interactions. In the absence of coupling between residues, prediction of mutational effects is possible by assuming their additivity.

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Introduction

The method of double-mutant cycles was first introduced into protein engineering in 1984 to detect interactions between residues in the active site of the tyrosyl-tRNA synthetase [1]. The method has been further developed during the past decade and applied to measure the strength of intramolecular and intermolecular interactions in proteins with known structure, to obtain structural information on proteins and protein–ligand complexes without known structure (including transition states for folding, binding and catalysis), and to predict mutational effects when additivity can be assumed. Certain aspects of double-mutant cycles have been reviewed previously [2–5]. In this review, I will emphasize the wide range of applications and the potential of this technique. I will also point out the conceptual relationship of double-mutant cycle analysis to methods in other disciplines.

Two residues in a protein, X and Y, are mutated separately and together to give rise to a cycle (Fig. 1) that comprises wild-type protein (P-XY), two single mutants (P-X and P-Y) and the corresponding double mutant (P). The change in free energy upon mutation of X, associated with a structural or functional property of the protein, may be expressed relative to wild-type as $\Delta G_{\text{P-XY}\rightarrow\text{P-Y}}$. Likewise, the change in free energy associated with this property upon mutation of X when Y has already been mutated is $\Delta G_{\text{P-X}\rightarrow\text{P}}$. The free energy changes $\Delta G_{\text{P-XY}\rightarrow\text{P-X}}$ and $\Delta G_{\text{P-Y}\rightarrow\text{P}}$ are similarly defined. If the effects of the mutations are not independent of each other, then $\Delta G_{\text{P-XY}\rightarrow\text{P-Y}} \neq \Delta G_{\text{P-X}\rightarrow\text{P}}$ and $\Delta G_{\text{P-XY}\rightarrow\text{P-X}} \neq \Delta G_{\text{P-Y}\rightarrow\text{P}}$. The free energy of coupling, $\Delta\Delta G_{\text{int}}$, between residues X and Y is given by:

$$\Delta\Delta G_{\text{int}} = \Delta G_{\text{P-XY}\rightarrow\text{P-Y}} - \Delta G_{\text{P-X}\rightarrow\text{P}} = \Delta G_{\text{P-XY}\rightarrow\text{P-X}} - \Delta G_{\text{P-Y}\rightarrow\text{P}} \quad (1)$$

The changes in free energy upon mutation, $\Delta G_{\text{P-XY}\rightarrow\text{P-Y}}$, $\Delta G_{\text{P-X}\rightarrow\text{P}}$, $\Delta G_{\text{P-XY}\rightarrow\text{P-X}}$ and $\Delta G_{\text{P-Y}\rightarrow\text{P}}$, cannot be determined directly from experiment. Instead, they are calculated by assuming:

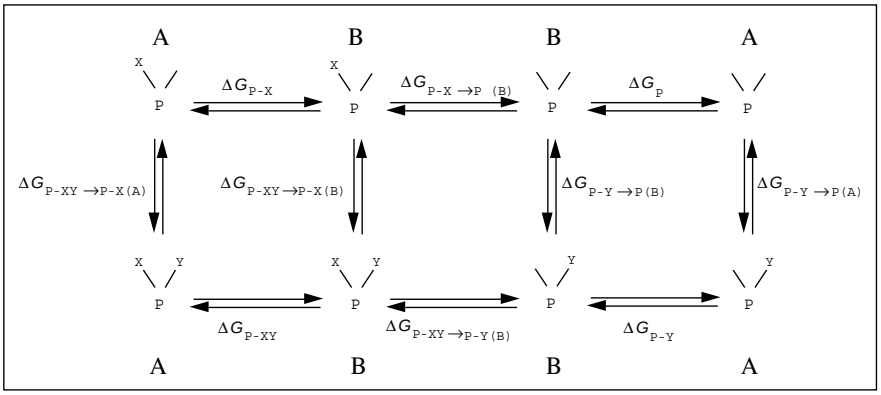
$$\Delta G_{\text{P-XY}\rightarrow\text{P-Y}} = \Delta G_{\text{P-XY}} - \Delta G_{\text{P-Y}} \quad (2)$$

$$\Delta G_{\text{P-X}\rightarrow\text{P}} = \Delta G_{\text{P-X}} - \Delta G_{\text{P}} \quad (3)$$

where $\Delta G_{\text{P-XY}}$, $\Delta G_{\text{P-Y}}$, $\Delta G_{\text{P-X}}$ and ΔG_{P} are the free energies of some process (e.g. binding or unfolding) that the proteins in the cycle are undergoing. Owing to the principle of free energy conservation in these cycles [6], the experimentally determined coupling energies are always for one state (B) relative to another (A) (Fig. 1). For example, the coupling energy between two residues is often measured in the folded relative to the unfolded state or in a protein–ligand complex relative to the unbound state. It is important to realize, therefore, that $\Delta\Delta G_{\text{int}} = 0$ does not necessarily imply that X and Y are not coupled to each other, but that there is no change in coupling energy on going from state B to state A.

The method of double-mutant cycles is related to classical genetic methods of identifying interacting proteins by means of suppressor mutations. It is also conceptually related [7] to the Hammett plot in physical organic chemistry. The Hammett equation [8] was originally formulated to describe the ionization of ring-substituted aromatic acids. The substitution and reaction sites in the aromatic ring are analogous to the two mutated positions in the protein sequence. The Hammett equation expresses the logarithm of the dissociation constant of the substituted ring as a product of contributions of the substitution and reaction sites, as follows:

Figure 1



Scheme for a double-mutant cycle. P is the protein and X and Y are the two residues in the protein that are being mutated. The cycle comprises wild-type protein (P-XY), two single mutants (P-X and P-Y) and the corresponding double mutant (P). $\Delta G_{P\text{-}XY\rightarrow P\text{-}Y}$, $\Delta G_{P\text{-}X\rightarrow P}$, $\Delta G_{P\text{-}XY\rightarrow P\text{-}X}$ and $\Delta G_{P\text{-}Y\rightarrow P}$ are the free energies corresponding to the appropriate mutations in the cycle. $\Delta G_{P\text{-}XY}$, $\Delta G_{P\text{-}Y}$, $\Delta G_{P\text{-}X}$ and ΔG_{P} are the measured free energy differences between two thermodynamic states, A and B, of the respective proteins in the cycle.

$$\log K_{XY} = \log K_{0Y} + \rho_Y \sigma_X \quad (4)$$

In this equation, X and Y stand for the substituent and acidic groups, K_{XY} and K_{0Y} are the respective dissociation constants of the substituted and unsubstituted molecules with the same acidic group Y, ρ_Y is the reaction constant and σ_X (= $\log(K_{X0}/K_{00})$) is the substituent constant. It can be readily shown that the case $\rho_Y = 1$ corresponds to the situation in which $\Delta\Delta G_{int} = 0$, i.e. the substitution and reaction sites are not coupled. Although the technique of double-mutant cycles is related to methods that have been in use for a very long time in other disciplines, it has been further developed and extended in many important respects in the field of protein engineering, as will be described below.

Analysis of intramolecular interactions in proteins with known structure

The strengths of different types of pairwise interactions in proteins have been determined using double-mutant cycles. An interaction of interest between two residues, X and Y, is identified in the structure. The two residues are then mutated singly and in combination and the free energy of unfolding of each protein in the cycle is measured, thus allowing one to calculate the coupling energy in the folded relative to the unfolded state. In ideal circumstances [9], where the mutations remove the interaction under study without adding new interactions or significantly perturbing the protein structure, subtraction of $\Delta G_{P\text{-}X\rightarrow P}$ from $\Delta G_{P\text{-}XY\rightarrow P\text{-}Y}$ causes the interaction energies of X and Y with the rest of the protein to cancel out. In such cases, the coupling energy is equal to the direct interaction energy between X and Y plus changes in solvation energy due to the mutations [9]. In order to minimize the possibility of new interactions being formed, interacting sidechains are usually replaced with alanine. There are cases, however, when it is not possible to use alanine as a reference mutant state. For example, buried residues may need to be replaced with relatively bulky amino acids in order to prevent formation of destabilizing cavities. Such 'not-to-alanine' double-mutant cycles can be decomposed into four double-mutant cycles with mutations only to alanine and the coupling energy for the 'not-to-alanine' cycle can be expressed as a function of the coupling energies of these four cycles [10].

The energies of different types of pairwise interactions in proteins have been measured using double-mutant cycles. Examples include electrostatic interactions between residues *i* and *i*+4 in α-helices which were found to contribute ≤ 0.5 kcal mol^{-1} to protein stability [9,11], an aromatic–aromatic interaction between residues *i* and *i*+4 in an α-helix which was found to contribute about 1 kcal mol^{-1} to protein stability [12], surface and buried salt-bridges which are worth 1–5 kcal mol^{-1} [13–16], and a charge–aromatic interaction worth about 1 kcal mol^{-1} [17]. An important issue is whether estimates of the energies of different types of interactions based on double-mutant cycle analysis in one system are transferable to other systems. Although the limited experimental data suggest that the answer to this is positive, much more experimental work needs to be carried out to establish whether this is indeed the case. The 'transfer' principle is in apparent conflict with the claim [18] that the free energy of a system cannot be decomposed in terms of specific pairwise interactions. Although it is clear that the free energy of a system is a sum of the free energies of its subsystems only if they are independent, it is an issue of debate [19,20] whether methods designed to isolate the contributions of specific interactions are invalid as claimed [18].

The method of double-mutant cycles has been extended to higher dimensions [21,22] to study cooperativity between interactions. For example, analysis of three interacting residues requires construction of a triple-mutant cube. In such a construct, the difference in coupling energies that correspond to opposite faces of the cube reflects the dependence of a pairwise interaction on a third

residue. Multidimensional mutant cycle analysis has been applied to the study of salt bridge networks in barnase [13], the Arc repressor [14] and λ repressor [15]. In the case of barnase, strong coupling was observed between two salt bridges formed by the triad of residues Asp8, Asp12 and Arg110. Each salt bridge was strengthened by about 0.8 kcal mol^{-1} when the other salt bridge was also present. In the Arc and λ repressors, such higher-order coupling was not observed. In general, the analysis of N interacting residues requires construction of the appropriate N-dimensional cube and is limited by the accuracy of measurements owing to the accumulation of experimental error.

Analysis of energetics of intermolecular interactions in proteins for which high-resolution structural information is available

Intermolecular interactions may be analyzed using double-mutant cycles that consist of the complex of the two wild-type proteins, the complex of each wild-type protein with a mutant protein, and the complex of the two mutant proteins. Thus, a series of n single mutations in one protein and m single mutations in another protein allow one to analyze by double-mutant cycles $n \times m$ potential interactions between the two proteins. The reference states in such cycles are the free proteins in solution which have a coupling energy of zero. This approach may be applied to any intermolecular interaction and not just to protein–protein interactions. For example, Steyaert *et al.* [23,24] analyzed the interaction between ribonuclease T_1 and dinucleoside phosphate substrates using double-mutant and triple-mutant cycles that consist of mutant proteins and modified substrates.

Double-mutant cycles have been employed extensively to study the stability and dimerization specificity of coiled-coils. Particular attention has been paid to the contribution of interhelical interactions between positions e and g [25–27], but the hydrophobic core of a coiled-coil was also recently analyzed in this manner [28]. Krylov *et al.* [25] established a thermodynamic scale based on double-mutant cycles for the relative contribution to leucine zipper stability of different pairs of residues commonly found at these positions. The most stable pair was found to be Glu–Arg with a $\Delta\Delta G_{int}$ of –1.14 kcal mol^{-1} in close agreement with the values for the strength of surface salt bridges in barnase determined by mutant cycle analyses [14]. In contrast, the Glu–Lys pair was found to contribute only –0.14 kcal mol^{-1} to the coiled-coil stability and the Glu–Glu pair was found to be destabilizing with a $\Delta\Delta G_{int}$ of +0.78 kcal mol^{-1}. These results are in agreement with the double-mutant cycle analyses carried out by Hodges and co-workers [26,27], but not with other work [29] based on pK_a shifts which indicated that interhelical electrostatic interactions at positions e and g contribute very little to coiled-coil stability. It has been argued [30] that these discrepancies are due to the fact that total pairwise interaction energies were measured [25–27] and not just their electrostatic components. The electrostatic component can be isolated by carrying out double-mutant cycle analysis at high salt where $\Delta\Delta G_{int}$ should be equal to zero if the interaction is only electrostatic and if shielding by salt is complete [9]. It has also been argued [30] that structural controls were not carried out to ensure that the mutations in these double-mutant cycle studies [25–27] did not cause conformational changes.

The most extensive double-mutant cycle analysis of protein–protein interactions carried out to date is for the barnase–barstar interface [31]. In this study, double-mutant cycles were constructed for a set of five barnase and seven barstar residues known to be involved in the interaction between these proteins. The strongest coupling energies (1–7 kcal mol^{-1}) were found between pairs of charged residues. Importantly, some of these strong coupling energies would not have been predicted by inspection of the structure. In general, it was found that the coupling energy decreased with increasing distance between the residues with only weak coupling being observed at distances greater than 7 Å. By measuring rate constants of association of barnase and barstar, it was also possible to characterize the transition state of association [31,32]. The effects of the mutations on the kinetics of association were mostly additive ($\Delta\Delta G_{int} = 0$), except for charged residues less than 10 Å apart, indicating that most of the interactions in the final complex are absent in the transition state.

Interpretation of coupling energies in the absence of structural information: docking and structure predictions

The information obtained from double-mutant cycles is similar in nature to that from nuclear Overhauser spectroscopy: two residues may be shown to be within an interaction distance. The major pitfall of this approach to structure determination is that strong coupling energies may reflect indirect interactions, whereas weak coupling energies may be due to compensations that arise from structural rearrangements. The appeal of this approach is that because it is based on kinetic and thermodynamic measurements it can be used to characterize structures that are inaccessible to NMR and X-ray crystallography, such as transition states of binding and folding, or structures that are difficult to study using these methods, such as membrane proteins. The uncertainty inherent to this method may be overcome, in part, by generating a large number of cycles so that false positives or false negatives may be disregarded. Prior biochemical or structural data that limit the number of positions that need to be mutated are extremely valuable to this approach.

An impressive example for this approach is the docking of a scorpion toxin peptide inhibitor to the Shaker potassium channel [33,34]. Initially [33], the extent of interaction between eight basic residues in the toxin and three

residues in the channel was determined. Out of the 24 coupling energies measured, only the one for the interaction between Arg24 in the toxin and Asp431 in the channel was outstanding. Other weak coupling energies were also detected. On the basis of this information and a constraint based on earlier biophysical studies, it was possible to generate a model for the interaction of these two molecules. This work has been extended recently by generating more mutants of both the toxin and the channel and analyzing their binding to each other [34]. It is important to point out that coupling energies of zero indicate which residues do not interact and are, therefore, also valuable for docking studies.

Another potential approach to prediction of interactions combines detection of correlated mutations by multiple sequence alignment with double-mutant cycle analysis. Correlated mutations at two (or more) positions indicate that the residues at these positions are coupled and may be in contact. Double-mutant cycles allow one to experimentally test whether residues at positions with correlated mutations are indeed energetically coupled. Such a study was carried out for GroEL [35]. Multiple sequence alignment of the hsp60 family of chaperonins showed that mutations at positions 138 and 519 correlate, i.e. the amino acid pairs Cys138–Cys519 and Val138–Ala519 were found significantly more than the other combinations. Double-mutant cycle analysis showed that the residues at these positions in GroEL are indeed energetically coupled although, as the crystal structure later showed, they are separated by a large distance. This study shows that correlated mutations together with double-mutant cycle analysis is a powerful tool but that structural interpretation of coupling energies should be performed with great caution, particularly in the case of allosteric proteins.

Double-mutant cycle analysis has been carried out extensively in recent years to characterize transition states of folding and folding intermediates. Rate constants of unfolding and refolding of the proteins in the cycles are measured, thereby allowing one to calculate pairwise and higher-order interaction energies in the transition state for folding and in folding intermediates. Examples for such analyses include barnase [22,36], chymotrypsin inhibitor 2 [37] and phosphoglycerate kinase [38]. Values of zero or one in ϕ-analysis of folding [39] indicate whether a region containing a mutation is as completely unfolded or folded in the transition state (or intermediate state) as in the unfolded or folded states, respectively. Fractional ϕ-values are difficult to interpret, but in conjunction with double-mutant cycles they provide structural information on such states at almost atomic resolution [37,40]. Double-mutant cycles can also be used to characterize changes in energies of pairwise or higher-order interactions as a function of the reaction coordinate of other processes such as enzyme catalysis [41,42] and allosteric transitions (A Aharoni, A Horovitz, unpublished data).

Additivity-based predictions of protein function

Additivity in mutational effects exists when $\Delta\Delta G_{int} = 0$, i.e. when the effect of a double mutation is equal to the sum of effects of the corresponding two single mutations. By assuming additivity, one may predict the free energy of a structural or functional property of any protein in the cycle from those of the three other proteins in the cycle. A striking case of additivity in protein–protein interactions is the interaction of ovomucoid third-domain inhibitors with elastase, chymotrypsin and subtilisin. Laskowski and coworkers [43] chose to study these interactions in an attempt to develop a sequence-to-reactivity algorithm that bypasses the folding problem. Additivity in this system was also demonstrated for data that correspond not only to double-mutant cycles but also to more complex mutational flow diagrams [44]. Using this approach, it was possible to predict the binding constants of ovomucoid third-domain inhibitors to the above-mentioned serine proteinases and to suggest a sequence for a new, more powerful and selective, ovomucoid third-domain inhibitor of subtilisin [44]. Additivity in mutational effects on protein function was also observed by carrying out an *in vitro* evolution experiment to test for correlated mutations [45]. 11 amino acids in the helix-turn-helix of λ repressor were replaced by alanine using a combinatorial procedure. Mutants were tested for activity in conferring immunity to *Escherichia coli* upon superinfection with the phage λ KH54. If mutation to alanine at a certain position is neutral with respect to activity, then its expected frequency at that position is 0.5. Comparison of the observed frequencies of pairwise mutations with the observed frequencies of the corresponding single mutations makes it possible to test for additivity in the mutational effects. Using such an additivity-based approach, the activity class of most double mutants could be predicted with 90% confidence.

Additivity has also been observed in mutational effects on stability of the gene V protein from bacteriophage *f*1 and its binding of single-stranded DNA [46]. The structural basis for the additivity in mutational effects on function was studied by determining the crystal structures of a series of single mutants and the corresponding double mutants [46]. The coordinate shifts in the double mutants were found to be nearly the sum of the coordinate shifts in the single mutants. The structural effects due to each single mutation were found to be localized to the site of mutation and have little effect on the other residue. Examination of the distance dependence of the coordination shifts showed that they are greatest near the sites of mutation and fall off rapidly, but not isotropically, with increasing distance. Beyond 10 Å, very few coordination shifts were observed, in agreement with the studies on the barnase–barstar complex [31,32]. Although the extent of coupling between two residues is generally found to decrease with increasing interresidue distance, the nonisotropic nature of structural effects of mutations may

sometimes lead to long-range coupling. Long-range coupling has also been attributed to propagation of electrostatic interactions through the low-dielectric medium of protein interiors [47,48]. Although long-range coupling is expected in the case of allosteric proteins, where long lines of communication exist [4,35], it has also been observed in many nonallosteric proteins [4]. It is present to a much lesser extent in rigid molecules such as the ovomucoid third-domain inhibitors [43,44].

Some future directions

Although much work has been done in recent years to measure the strength of different types of stabilizing interactions in proteins by using double-mutant cycles, there are still too few data obtained in this manner to determine whether the 'transfer' principle is valid. Integration of experimental scales of helix propensities determined using different systems has shown that true propensities can be separated from context-dependent effects. Hopefully, the accumulation of more data on interactions using the double-mutant cycle approach will eventually lead to an inventory of the energetics of different interactions in proteins that is context-independent. There are also few data, so far, on intermolecular interactions obtained using double-mutant cycles. The method is yet to be applied to many other types of interaction, such as protein–DNA, drug–DNA and protein–lipid interactions. In addition, there have not yet been many studies on cooperativity in intermolecular interactions. One outstanding example for this approach is the multidimensional cycle analysis of ATP binding to the tyrosyl-tRNA synthetase [42].

Not many examples exist, as yet, for ligand docking or protein structure predictions using double-mutant cycles. Candidates for such experiments tend to be systems that are difficult to study using NMR and X-ray crystallography. It is necessary, however, to compare structures obtained using the double-mutant cycle procedure with structures determined by NMR or X-ray crystallography in order to validate this approach and understand its limitations. The method should, therefore, also be applied to systems that are accessible to NMR or X-ray crystallography to facilitate such a comparison.

Further theoretical work needs to be carried out on the validity of the double-mutant cycle approach in isolating energetic contributions to folding and stability. Theoretical work is also required on the type and number of double-mutant cycle derived constraints required for docking and structure prediction.

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