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New Concepts

Inverse Thinking about Double Mutants of Enzymes†

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ABSTRACT: The quantitative effect of a second damaging mutation on a mutated enzyme may be additive, partially additive, synergistic, antagonistic, or absent, in the double mutant. Each of these five possible types of interactions has its own mechanistic explanation [Mildvan, A. S., Weber, D. J., and Kuliopulos, A. (1992) *Arch. Biochem. Biophys. 294*, 327−340]. Additive effects indicate independent functioning of the two residues in the process being studied, such as catalysis (k_{cat}) or substrate binding (K_S). Departures from additivity reflect interaction of the two residues. Thus, partial additivity indicates cooperativity, synergy indicates anticooperativity, and antagonism indicates opposing structural effects of the two mutations. No additional effects represent limiting cases of either partial additivity or antagonism. A significant conceptual simplification is achieved by applying inverse thinking, namely, by using the parameters of the double mutant rather than those of the wild-type enzyme as the reference point. To explain partially additive effects on k_{cat}, inverse thinking starts with the k_{cat} of the double mutant. Restoring only one residue increases k_{cat} by the factor A. Restoring only the other residue increases k_{cat} by the factor B. Restoring both residues is shown to increase k_{cat} by a factor greater than $A \times B$, with the excess directly measuring the cooperativity. Similarly, inverse thinking provides simpler and more intuitive explanations of synergistic and antagonistic effects, as illustrated by specific examples.

Mutations of active site residues of enzymes usually introduce free energy barriers to the binding of substrates, transition states, or both, thereby damaging catalysis. In a quantitative treatment of the effects of double mutations of enzymes in terms of the free energy barriers introduced to substrate binding (K_S) or to catalysis (k_{cat}) (*1*), we pointed out that mathematically, the effect of the second mutation could interact with the effect of the first mutation in one of only five possible ways in the double mutant: additive, partially additive, synergistic, antagonistic, or no additional effect. Because free energy barriers to binding or to catalysis (which are logarithms of K_S or k_{cat}) were being added, we were comparing the product of the effects on these parameters of the two single mutants with the effect of the double mutant.[1] Mechanistic explanations for each of these five possible interactions on both equilibrium constants and rate constants were provided (*1*), and these concepts have been applied to many systems (*2*−*12*).

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[1] We have previously noted that k_{cat}/K_m, while able to be treated by this analysis (*1*−*3*), generally contains more rate constants than k_{cat} because k_{cat} does not include rate constants for the binding and dissociation of substrates. For this reason, the effects of mutations on k_{cat} can, in certain circumstances, better discriminate the underlying mechanism than can effects on k_{cat}/K_m (*3*). Hence, effects of mutations on k_{cat} and K_s only are considered in this article, although effects on k_{cat}/K_m have been treated theoretically (*3*) and experimentally (*2*).

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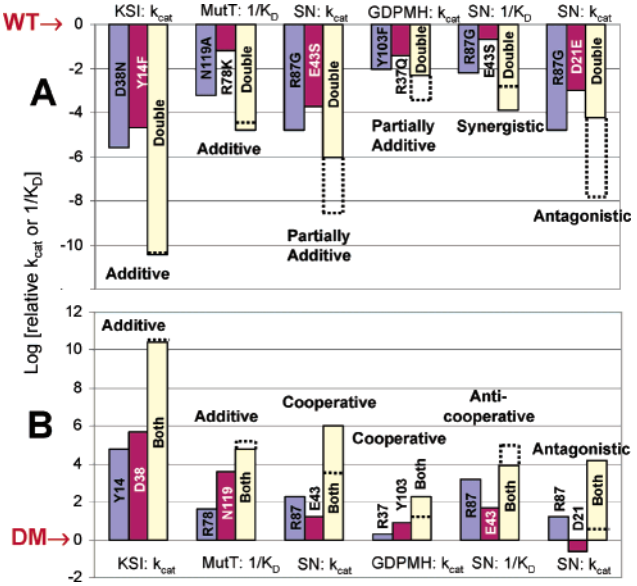

FIGURE 1: Interactions of the effects of two single mutants on catalysis (k_{cat}) or substrate binding ($1/K_D$) in double mutants of enzymes. (A) Traditional analysis with reference to wild-type (WT) parameters showing decreases in k_{cat} or in $1/K_D$ in single and double mutants (*1*). The dotted bars indicate the product of the effects of the two single mutants, the result expected for simple additivity. (B) Inverse analysis with reference to the parameters of the double mutant (DM), showing the effects of restoring wild-type residues individually and in pairs. The dotted bars indicate the product of the effects of restoring the two residues individually, the result expected for simple additivity. In panels A and B, parameters of the following enzymes are shown: KSI, ketosteroid isomerase (*3*); GDPMH, GDP-mannose mannosyl hydrolase (*6*, *19*); MutT, MutT nucleoside triphosphate pyrophosphohydrolase (*4*, *5*, *15*); SN, staph nuclease (*5*, *7*).

The simplest cases involve additive effects in the double mutant. For example, additive effects of two damaging single mutations on k_{cat}, in the double mutant, indicate that the two residues act independently in promoting the same rate-limiting step, an explanation which is intuitively reasonable, and usually occurs when the mutated residues are far apart in the structure (i.e., the distance between the nearest side chain heavy atoms is $\geq$8 Å) (*1*, *2*). Such behavior was found with ketosteroid isomerase (*3*), where the D38N and Y14F single mutations of the base and acid catalysts decreased k_{cat} by factors of $10^{5.6}$- and $10^{4.7}$-fold, respectively, while the double mutant decreased k_{cat} by $\geq 10^{10.4}$-fold (Figure 1A). The carboxylate Oδ atom of D38 and the Oη atom of Y14 were, on average, 8.0 Å apart in the NMR structure of the enzyme−steroid complex (*13*).

Additive effects of two damaging single mutations on dissociation constants indicate that the two residues act independently in promoting binding. A recent example is provided by the MutT enzyme which tightly binds the oxidatively damaged nucleotide 8-oxo-dGMP with a K_D of 52 nM (*14*). In the solution structure of the MutT−Mg^{2+}−8-oxo-dGMP complex, the nearest side chain nitrogens of N119 (Nδ) and R78 (Nη) are 8.4 Å apart, and both are hydrogen bonded to 8-oxo-dGMP (*15*). The N119A and R78K single mutants increased this K_D of 8-oxo-dGMP by factors of $10^{3.2}$- and $10^{1.2}$-fold, respectively, while the double mutant increased this K_D by $10^{4.8}$-fold, indicating additive effects, within experimental error (*4*). Because additive effects are simple and intuitive, they do not require "inverse thinking" (Figure 1B) for clarification of their mechanisms.

Partially additive effects on K_S or k_{cat} in a double mutant are defined as showing less damage than the product of the damaging effects of the two single mutants, but more damage than either of the two single mutants alone. Partially additive effects in the double mutant indicate partially cooperative effects of the two residues in promoting binding (K_S) or catalysis (k_{cat}) (*1*). This can be explained by arguing that each single mutant abolishes both an intrinsic noncooperative contribution and the same cooperative contribution to binding (or catalysis). The double mutation abolishes the noncooperative contributions of both residues, but abolishes the cooperative contribution only once. Hence, the ratio of the product of the effects of the two single mutants (which counts the cooperative component twice) to that of the double mutant (which counts the cooperative component once) measures the cooperative contribution of the two residues to binding (or catalysis) (*1*). For example, with Staph nuclease, the R87G mutant (which removed a Lewis acid) decreased k_{cat} by $10^{4.8}$-fold, the E43S mutant (which removed a general base) decreased k_{cat} by $10^{3.7}$-fold, and the double mutant decreased k_{cat} by only $10^{6.0}$-fold, rather than $10^{8.5}$-fold (*5*) (Figure 1A). Hence, R87 and E43 act both independently and cooperatively, with the cooperative component contributing a $10^{2.5}$-fold increase to catalysis. In several X-ray structures (Protein Data Bank entries 1SNQ, 1STA, and 1ENC), the nearest inter-side chain distance between R87 (Nη) and E43 (Oϵ) ranges from 7.1 to 7.5 Å.

Unlike the cases of simple additivity, this explanation of partial additivity is not intuitively obvious. In cases involving interacting residues, a significant conceptual simplification can be achieved by applying the power of inverse thinking, namely, by using the parameters of the double mutant rather than those of the wild-type enzyme as the reference point (Figure 1B). In the example of Staph nuclease described above, the k_{cat} of the R87G/E43S double mutant is $10^{6.0}$-fold lower than that of the wild type. Restoring only R87 to the double mutant (as in the E43S single mutant) increases k_{cat} by $10^{2.3}$-fold (to $10^{3.7}$-fold below that of the wild type). The value of $10^{2.3}$ directly measures the intrinsic, noncooperative contribution of R87 to k_{cat}, because E43 is absent. Restoring only E43 to the double mutant (as in the R87G single mutant) increases k_{cat} by $10^{1.2}$-fold (to $10^{4.8}$-fold below that of the wild type) which directly measures the noncooperative contribution of E43 to k_{cat}, because R87 is absent. Restoring both R87 and E43 to the double mutant (as in the wild-type enzyme) increases k_{cat} by $10^{6.0}$-fold which exceeds the product of the effects of individually restoring R87 and E43 ($10^{2.3} \times 10^{1.2} = 10^{3.5}$) by $10^{2.5}$-fold, the cooperative contribution to k_{cat}. Thus, the inverse analysis directly measures the intrinsic, noncooperative contributions of the individual residues to k_{cat}, and their cooperativity. It also shows the cooperative component to increase k_{cat}, consistent with intuition (Figure 1B). Such inverse thinking is reminiscent of research involving the synthesis of organic catalysts by successive addition of functional groups to cyclodextrins and to other nonenzymatic scaffolds (*16*−*18*).

A recent example of partially additive effects on k_{cat} is provided by GDP-mannose mannosyl hydrolase. Both R37 and Y103, the side chain Nη and Oη atoms of which are only 5.0 Å apart in the X-ray structure (*19*), promote the

departure of the GDP leaving group by donating hydrogen bonds to the same (β) phosphate. The R37Q mutant decreased k_{cat} by $10^{1.4}$-fold; the Y103F mutant decreased k_{cat} by $10^{2.0}$-fold, and the R37Q/Y103F double mutant decreased k_{cat} by only $10^{2.3}$-fold rather than $10^{3.4}$-fold, indicating a cooperative contribution of $10^{1.1}$-fold to catalysis (*6*) (Figure 1A). Applying inverse thinking (Figure 1B) shows that the k_{cat} of the double mutant is $10^{2.3}$-fold lower than that of the wild-type enzyme. Restoring only R37 (as in the Y103F mutant) increases k_{cat} by $10^{0.3}$-fold. Restoring only Y103 (as in the R37Q mutant) increases k_{cat} by $10^{0.9}$-fold. Restoring both R37 and Y103 (as in the wild type) increases k_{cat} by $10^{2.3}$-fold, more clearly measuring the cooperative contribution to catalysis as the extra $10^{1.1}$-fold.

Synergistic effects of two damaging mutations are defined by more damage in the double mutant, in comparison with the product of the effects of the two single mutants. Synergistic effects indicate anticooperative interaction of the two residues in the process that is being investigated (*1*). For example, the R87G mutant of Staph nuclease weakens the binding (i.e., increases the K_S) of the Ca^{2+}pdTdA substrate to the enzyme by $10^{2.2}$-fold, the E43S mutant weakens substrate binding by $10^{0.7}$-fold, while the double mutant weakens binding by $10^{3.9}$-fold, which is 10-fold greater than the effect of simple additivity (Figure 1A) (*5*). If inverse thinking is used directly (Figure 1B), the double mutant binds Ca^{2+}pdTdA $10^{3.9}$-fold weaker than the wild type. Restoring only R87 to the double mutant strengthens substrate binding by $10^{3.2}$-fold (as in the E43S single mutant). Restoring only E43 to the double mutant strengthens substrate binding by $10^{1.7}$-fold (as in the R87G single mutant). Restoring both residues to the double mutant strengthens binding by $10^{3.9}$-fold, which is 10-fold less than the product of the strengthening effects of the two single mutants ($10^{3.2} \times 10^{1.7} = 10^{4.9}$). Hence, a factor of 10 in the binding affinity ($1/K_S$) for Ca^{2+}pdTdA has disappeared when both R87 and E43 are restored (Figure 1B), possibly due to strain between these residues in binding the substrate, resulting in anticooperativity. This factor of 10 (corresponding to a 1.34 kcal/mol increase in binding free energy) measures the strain or anticooperativity, and the factors of $10^{3.2}$ and $10^{1.7}$ measure the intrinsic, noncooperative contributions of R87 and E43, respectively, to substrate binding (Figure 1B).

Antagonistic effects of two mutations on binding (K_S) or catalysis (k_{cat}) are defined by less damage in the double mutant than in the more damaged single mutant. In such cases, the second damaging mutation has partially repaired the damaging effect of the first mutation (*1*). Antagonistic effects result from opposing structural effects of the two mutations on binding or catalysis. An example is provided by the effects on k_{cat} of the R87G (Lewis acid) and D21E (metal ligand) mutants of Staph nuclease (*7*), the respective Nη and Oϵ atoms of which are 5.0−5.4 Å apart in two X-ray structures (PDB entries 1SNQ and 1STA). The R87G mutant decreased k_{cat} by $10^{4.8}$-fold, the D21E mutant by $10^{3.0}$-fold, and the R87G/D21E double mutant by only $10^{4.2}$-fold, slightly repairing the damaging effect of the R87G mutant. The $10^{3.6}$-fold departure from additivity measures the antagonistic effects of these mutations on k_{cat} in the double mutant (Figure 1A). Applying inverse thinking is helpful in this case (Figure 1B). Thus, the double mutant is $10^{4.2}$-fold less active than the wild type. Restoring only R87 increases k_{cat} by $10^{1.2}$-fold, while restoring only D21 decreases k_{cat} by $10^{0.6}$-fold. Restoring both R87 and D21 increases k_{cat} by $10^{4.2}$-fold, which is $10^{3.6}$-fold greater than the product of the effects of restoring R87 and D21 individually ($10^{1.2} \times 10^{-0.6} = 10^{0.6}$), directly measuring the antagonism. While reminiscent of cooperativity, antagonism differs from cooperativity since the effect of adding the second mutation (D21E) partially repaired the effect of the first one (R87G) in the double mutant (Figure 1A). Stated inversely, restoring only D21 to the double mutant decreased k_{cat}, while adding R87 (to form the wild type) antagonistically increased k_{cat}.

No additional effect of a second damaging mutatation on K_S or k_{cat} of a mutated enzyme represents a limiting case of either partial additivity or antagonism (*1*), which have already been discussed. In all cases, departures from simple additivity, in terms of free energy, measure the amount of cooperativity, anticooperativity, or antagonism between the effects of the two mutated residues.

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