Negative Epistasis and Evolvability in TEM-1 β-Lactamase—The Thin Line between an Enzyme's Conformational Freedom and Disorder

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Abstract

Epistasis is a key factor in evolution since it determines which combinations of mutations provide adaptive solutions and which mutational pathways toward these solutions are accessible by natural selection. There is growing evidence for the pervasiveness of sign epistasis—a complete reversion of mutational effects, particularly in protein evolution—yet its molecular basis remains poorly understood. We describe the structural basis of sign epistasis between G238S and R164S, two adaptive mutations in TEM-1 β-lactamase— an enzyme that endows antibiotics resistance. Separated by 10 Å, these mutations initiate two separate trajectories toward increased hydrolysis rates and resistance toward second and third-generation cephalosporins antibiotics. Both mutations allow the enzyme's active site to adopt alternative conformations and accommodate the new antibiotics. By solving the corresponding set of crystal structures, we found that R164S causes local disorder whereas G238S induces discrete conformations. When combined, the mutations in 238 and 164 induce local disorder whereby nonproductive conformations that perturb the enzyme's catalytic preorganization dominate. Specifically, Asn170 that coordinates the deacylating water molecule is misaligned, in both the free form and the inhibitor-bound double mutant. This local disorder is not restored by stabilizing global suppressor mutations and thus leads to an evolutionary *cul-de-sac*. Conformational dynamism therefore underlines the reshaping potential of protein's structures and functions but also limits protein evolvability because of the fragility of the interactions networks that maintain protein structures.

Epistasis, that is, nonadditive effects of mutations, determines the topography of fitness landscapes [1,2]. Hence, epistasis describes which combinations of mutations provide adaptive solutions and which stepwise mutational pathways leading to these solutions are selectively accessible [3–6]. In doing so, epistasis determines the capacity of organisms, and of their proteins, to evolve and adapt, that is, their evolvability. Interestingly, by increasing the dependence of mutational pathways on early mutations, epistasis causes mutational pathways to be historically contingent upon initial mutations [7,8]. This influence is particularly strong in case of sign epistasis, where the sign-of-the-fitness effect of a mutation (beneficial or deleterious) depends on its genetic background [3]. For example, mutations with individual beneficial effects, but combined deleterious effect, show reciprocal sign epistasis. They create a rugged adaptive landscape, where different trajectories may lead to adaptive peaks of different

heights [1,3,4,9–11] and whereby some of the trajectories can turn into evolutionary dead ends, or *cul-de-sacs* [12–14].

Epistasis, including sign epistasis, is pervasive [2,3]. The phenomenon itself is measured and relates to organismal fitness. It is clear, however, that its origins lie in antagonistic interactions between mutations, either in separate genes (or proteins) or within the same gene/protein. However, the molecular basis of epistasis remains poorly understood [10]. We have better understanding of positive epistasis at the single gene level, such as in deer mouse hemoglobin adapted to high altitude [15]. Typically, stabilizing mutations, which have no effect on protein fitness on their own, are revealed as beneficial when they compensate for new function mutations that are typically destabilizing [16]. Such stabilizing mutations can have local, specific compensatory effects [17,18], or global effects, when they can compensate for a whole range of destabilizing mutations [16] in non-contacting residues [19–21]. One case study of positive epistasis in the vertebrate glucocorticoid receptor revealed a mutation that was initially neutral, yet by reorienting an α-helix, it enabled the acceptance of an adaptive mutation that would be deleterious on its own [22]. Overall, it appears that stabilization of the protein's configuration promotes its adaptive potential and underlines positive epistasis [21,22]. However, a detailed, structural understanding of negative sign epistasis is lacking: Why do certain combinations of mutations, each of which is beneficial individually, become deleterious when combined [23,24]?

Here, we explored a case of negative reciprocal sign epistasis between two adaptive mutations in TEM-1 β-lactamase, an enzyme present in numerous antibiotic-resistant bacteria. These studied mutations, G238S and R164S, are among the most common mutations in clinically isolated variants and therefore represent a highly relevant case study [25,26]. Wild-type TEM-1 confers resistance to natural penicillin antibiotics. However, in the clinic, strains with new resistance mutations are frequently isolated. This increased resistance is the outcome of adaptive evolution of TEM-1 for new, so-called second and third-generation cephalosporins antibiotics. Adaptive evolution of TEM-1 has occurred in numerous parallel events and can be readily reproduced in the laboratory [25]. The increased catalytic activity of TEM-1 to second and third-generation cephalosporins antibiotics therefore comprises a broadly accepted model for enzyme evolution [3,25,26]. Owing to the large sampling of TEM-1 sequences by natural and directed evolution, the absence of certain combinations of mutations likely indicates their negative epistatic interaction. This is the case with G238S and R164S, which were individually found in 91 and 51 different variants, respectively, both in laboratory and in clinical contexts [25]. However, their combination has only been observed in one clinical sample [27]. Indeed, the combination of G238S and R164S results in *in vivo* resistance levels to cefotaxime that are lower than each of the single mutants (4-fold lower than R164S, and 8-fold lower than G238S) and are similar to wild-type TEM-1 (Refs. [7] and [27]). Laboratory experiments attempting to evolve variants carrying the R164S mutation toward high cefotaxime activity indicated that R164S blocks the path to maximal cefotaxime degradation activity [7]. One mutation, E104K, improves R164S's activity, but the activity of the R164S/E104K double mutant was still inferior to G238S/E104K [24]. The only traceable trajectory to higher cefotaxime resistance in these laboratory evolution experiments was through reversion of R164S and take over by G238S (Ref. [7]). Thus, R164S seems to comprise an evolutionary *cul-de-sac* [7]. However, the molecular basis underlining the limited adaptive potential of R164S and of the reciprocal sign epistasis with G238S remains unclear.

The strong, nonadditive interaction between R164S and G238S is surprising for two reasons: firstly, the mutation sites are not in direct contact (>10 Å apart), and secondly, combining these mutations does not further compromise TEM-1's global configurational stability [7,16]. To determine the structural basis for this classical case of reciprocal sign epistasis, and to understand why R164S might lead to an evolutionary *cul-de-sac*, we solved the crystal structures of the R164S, G238S and double mutant R164S/G238S, in their free forms and bound to an inhibitor. Although these mutations are key first-step mutations in the adaptation of TEM-1 to second and third-generation cephalosporins antibiotics, the structure of enzymes harboring these mutations with no additional active-site mutations has not been available thus far. The combination of free *versus* inhibitor bound and of variable temperature X-ray data allowed for increased sampling of the conformational ensemble of TEM-1, thus unraveling the structural basis of negative epistasis and evolvability in TEM-1.

Results

Mutations that confer TEM-1 with the ability to hydrolyze new antibiotics are located mostly on the loops surrounding the active site: the "Ω-loop" (residues 164–179, Ref. [28]), the "238-loop" (residues 238–242, Ref. [29]) and the "101-loop" (residues 101–111, Ref. [30]). The mutations studied here, G238S and R164S, enhance cefotaxime hydrolysis (third-generation cephalosporins antibiotic) and reside on the 238-loop and the Ω-loop, respectively [7,29]. Mutation G238S increases cefotaxime resistance levels by ~16-fold: the MIC or the minimal inhibitory concentration increases from 0.06 μg/ml to 1 μg/ml (Table 1). Mutation R164S