Global analysis of multi-mutants to discover stabilizing amino acid substitutions

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Abstract

The identification of stabilizing amino acid substitutions in proteins is a key challenge in protein engineering. Advances in biotechnology have enabled assaying thousands of protein variants in a single high-throughput experiment, and more recent studies use such data in protein engineering. We present a Global Multi-Mutant Analysis (GMMA) that exploits the presence of multiply-substituted variants to identify individual substitutions that stabilize the functionally-relevant state of a protein. GMMA identifies substitutions that stabilize in different sequence contexts that thus may be combined to achieve improved stability. We have applied GMMA to >54,000 variants of green fluorescent protein (GFP) each carrying 1-15 amino acid substitutions. The GMMA method achieves a good fit to the data while being analytically transparent with a physical interpretation of the estimated parameters. The six top-ranking substitutions are demonstrated to progressively enhance GFP and in general, our analysis recovers nearly all the substitutions previously reported to be beneficial for GFP folding and function, only using a single experiment as input.

Introduction

A major challenge in practical applications of proteins is the engineering of protein stability while at the same time maintaining the function of the protein. New developments in biotechnology are continuously applied to address this challenge with high-throughput methods currently in focus. Synthesis, screening and sequencing may today be performed for thousands of protein variants in parallel via *Multiplexed Assay of Variant Effects* (MAVE) also known as *deep mutational scanning* [1, 2]. Such experiments can identify loss-of-function variants with high accuracy, but are often unable to gauge more subtle effects on stability. Thus, stabilizing, neutral or mildly destabilizing substitutions are likely to have a minor, if any, detectable impact on protein function and are therefore more difficult to identify from such experiments. Additive contributions to stability or catalysis are particularly attractive for protein engineering [3,4] and the ability to extract such from MAVE experiments could impact on the field substantially. Stabilizing and folding-enhancing substitutions may also provide robustness to other mildly destabilizing substitutions and thus enhance evolvability towards novel functions [5].

When a protein library contains doubly- or multiply-substituted variants, statistical models have been used to investigate how the effect of individual substitutions combine. One important technical consideration is the mathematical framework to capture (non-)additivity of the variant effects. For example, one would often find that the thermodynamic stability effects of two substitutions are additive when quantified by free energy differences, whereas the corresponding equilibrium constants would not be additive, but rather multiplicative. Thus, more general models typically consider additive single-substitution effects in a generalized latent space, which is constructed to maximize additivity, and transforms the sum of these to the assayed quantity via various non-linear functions to describe the data generated by a MAVE [6-8]. One study further showed that a thermodynamic model could be used to quantify the effects of single-substitutions on protein binding and structural stability from experimental measurements of the effects of both single and double mutants on binding [9]. This model was later shown to capture structural stability accurately [10] and a similar approach was successfully applied to fit deleterious effects of multiply-substituted variants [7].

Here, we present a Global Multi-Mutant Analysis (GMMA), that enables the identification of amino acid substitutions that have a general stability-enhancing effect with little dependency on the

sequence context, and thus substitutions with the potential to be additively combined. We demonstrate that these single-substitution effects may be informed by multiply-substituted protein variants, here referred to as multi-mutants. Along with the model, we present a biophysical interpretation that allows for a detailed analysis of fitted parameters and uncertainties. The central idea is to identify stabilizing substitutions by their ability to compensate other destabilizing substitutions. Specifically, while a stabilizing substitution may not have a measurable effect in the background of an already stable protein, it can be identified in the background of one or more destabilizing substitutions. The concept is similar to the "partner potentiation" principle formulated previously [11], but here generalized to multi-mutants and with a method to understand and handle the parameter estimation challenges that arises with random multi-mutant libraries.

We have applied our analysis to an experiment that reports the fluorescence of >54,000 variants of green fluorescent protein (GFP) each containing 1–15 of the total of 1,879 unique single-amino-acid substitutions observed in the experiment [6]. The GFP variants were generated using error-prone PCR (epPCR) and expressed in fusion with a red-fluorescent protein to correct for variations in expression levels. Then, fluorescence activated cell sorting (FACS) was used to divide the cells into eight fractions based on their level of green fluorescence, and each fraction was sequenced to link an intensity of fluorescence with a genotype. We have applied GMMA to the results of this single experiment to estimate the effects on stability of 1,107 single-substitutions, and identify stabilizing substitutions that do not compromise function and are directly applicable to engineering studies. As validations of the approach, we show that the model accurately predicts experimental variant effects that were not used in training, and show that the top six substitutions may progressively be combined in new GFP variants that achieve a more than 6-fold enhancement of cellular fluorescence compared to the starting point when expressed in *E. coli* cells. Finally, we show that the method identifies a large number of substitutions that have previously been described to be beneficial for improving GFP.

Results and Discussion

Biophysical concept

The identification of stabilizing single-amino-acid substitutions in an already stabile protein, when based on a functional assay, is challenging because these may not substantially improve the assayed function. Also, stability assays based on structural properties, e.g. via circular dichroism, may not directly report or help identify the particular state of the protein that is relevant for function. In order to address this situation, we define the states of a protein by a specific assayed property and consider an equilibrium between the active state, A, and an inactive state, D:

$$A \rightleftharpoons D \tag{1}$$

In analogy with traditional measurements of protein stability [12], we can probe this equilibrium by perturbing the system and measuring a deactivation transition. In the context of protein engineering the relevant change is to the protein sequence. Thus, we consider a "variable protein" and probe the stability related to Eq. 1 in amino acid sequence space. With this, the equilibrium does not describe a particular protein variant but rather a system that may be perturbed by changing the sequence of the protein.

The equilibrium is associated with a free energy of activation, denoted $\Delta G_v = G_v^A - G_v^D$ for a variant $v \in \{1 \dots V\}$ in a library of V amino acid sequence *variants* that will each contain one or more

individual amino acid *substitutions*. From this free energy of activation, here simply referred to as the stability, we can calculate the fraction of active protein which is assumed to be proportional to the functional readout (see Methods). This probing of the equilibrium in sequence space has some analogy to conventional denaturation experiments where the equilibrium of a "fixed protein" is probed by changes e.g. in temperature or solvent composition [12]. We stress that the term "stability" here refers specifically to the equilibrium in Eq. 1 and may not be the same as the thermodynamic folding stability, depending on the mechanism of inactivation.

The equilibrium Eq. 1 implies that amino acid substitutions stabilize or destabilize the protein via mechanisms that allow for compensation by other substitutions. Such effects are often found to be mostly independent and with additive free energies [11, 13, 14]:

$$\Delta G_v = \Delta G_{\mathrm{wt}} + \sum_{s \in v} \Delta\Delta G_s \tag{2}$$

Here, ΔG_{wt} is the stability of the "wild type" (or reference) sequence and $\Delta\Delta G_s$ is the stability effect of the substitution s. We note that our approach is not limited to additive effects and that couplings may be included in Eq. 2, as long as the data warrants estimation of these. Rather, the additivity in this particular model could be viewed as the desired output: We wish to identify those stabilizing substitutions that can compensate several different destabilized variants and thus be stabilizing additively in diverse backgrounds (Fig. 1a). This formulation is similar to previous global models [6-9, 14], but here applied to identify stabilizing substitutions and with a thermodynamic interpretation.

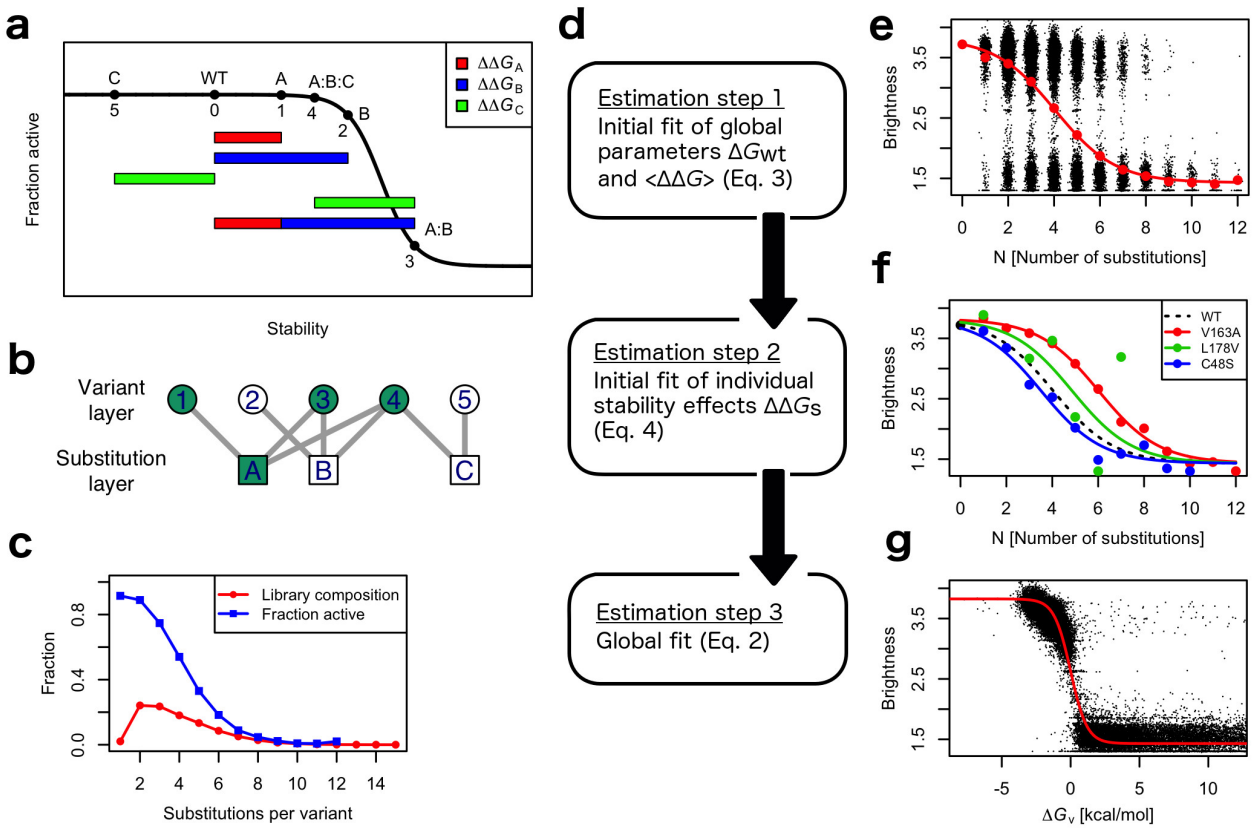

Figure 1. **(a)** A schematic outline of the GMMA approach. Consider a protein (WT) with five variants (named 1–5) that are composed of one or more of three substitutions (named A–C). The lengths of the colored bars represent the magnitude of the additive stability effects of these three substitutions. All single-substitution variants show wild-type-like activity, with the most destabilized variant, B, only being slightly less active than the wild type. While both variants A and B are active, the double mutant A:B is inactive. Thus, we infer the additive effect of A and B are both mildly destabilizing. Substitution C on its own does not appear to affect activity. However, when it is introduced into the inactive A:B background (to form A:B:C) it is able to compensate the loss of function, and we thus infer that substitution C is stabilizing with the magnitude of the green bar. (**b**) The five variants and the three substitutions may be represented in a bipartite graph, and as an example we highlight (in green) the subset of protein variants used to estimate the effect of substitution A. (**c**) Multi-mutant composition of the GFP library (red) shown together with the fraction of active variants (blue). The high population of variants in the transition region between 2 and 6 amino acid substitutions makes this excellent for GMMA. **(d)** Three-step estimation strategy. The three steps relate to panels e, f and g respectively **(e)** Fit of initial global parameters (red line) to the average brightness of variants with a given number of substitutions (red points) using Eq. 3. The GFP library is shown with random jitter in the horizontal coordinate to ease visualization (black dots). **(f)** Three examples of initial fits of individual stability effects (colored lines) compared to the WT fit from panel e (dashed line). Each $\Delta\Delta G_s$ is fit to the average *N*-mutant brightness (points) calculated only using the subset of variant that contains *s*. Mildly destabilizing substitutions, like C48S, lowers the endurance towards additional generally destabilizing substitutions and thus the system inactivates with fewer substitutions and the curve shifts left (blue line) compared to the wild-type (dashed line). On the other hand, stabilizing substitutions, like V163A, may be identified by their ability to increase the endurance towards substitutions, thus shifting the curve to the right (red line). Substitutions observed in few variants are difficult to fit, here demonstrated with L178V observed in only 14 variants compared to 677 and 194 variants for V163A and C48S respectively. **(g)** Result of the final global optimization of all stability effects (red line) to all variants (black dots).

The GMMA thus comprises a set of V equations, each describing the activity of a variant, and carrying a number of parameters, $\Delta\Delta G_s$, together with the global wild-type stability, ΔG_{wt}, and baseline parameters. For such a system of equations, it is important to have more data (variants) than parameters (substitutions), and this is possible with a multi-mutant library where a set of unique substitutions may be mixed in many different ways to make a larger set of variants. It is also important that all equations are coupled, and this may be tested by analyzing an undirected bipartite graph in which the protein variants constitute one layer of nodes (Fig. 1b, circles) and the unique substitutions the other layer (Fig. 1b, squares). This substitution-variant-graph formalism may be used to study many aspects of the multi-mutant library (see supplementary Appendix 1). For example, the degree distribution of variant nodes gives the distribution of the number of substitutions in the variants (Fig. 1c), which shows that most variants contain two to six substitutions. The fraction of active variants per N-mutant (Fig. 1c, blue line) shows that the substitutions are in general destabilizing and that the stability of GFP is approximately 4 when measured in units of "general substitution effects". GMMA identifies stabilizing substitutions by compensation of this general destabilization, and it is important that the variant library has an N-mutant distribution that covers the transition region where the system loses activity.

The inactive state, D, results from amino acid substitutions that are deactivating by reversible means, irrespective of mechanism, and is thus somewhat broadly defined. On the other hand, we term deactivating substitutions that cannot be compensated by stabilizing substitutions, e.g. removal of a crucial functional side chain, as *irreversible fatal substitutions* (IFS). IFS may be related to the assayed function of the protein, stability or folding hotspots, or for GFP, related to the chromophore maturation reaction. Since they are dominantly deleterious for the active system, IFS will artificially appear as highly destabilizing although nothing may be inferred about their effect on conventional structural stability. This highlights a distinct advantage that GMMA will only identify substitutions that stabilize the active system defined by the assay.

In the interpretation of the GFP data it is important to realize that the destabilization-via-substitutions is qualitatively different from conventional *in vitro* unfolding experiments where unfolding is induced after the irreversible chromophore maturation. Indeed, one of the best-known enhanced variants of GFP is named *superfolder GFP* (sfGFP) and this name refers to the ability to fold and mature before creating non-fluorescent inclusion bodies [15]. For GMMA, the considered equilibrium, Eq. 1, may likewise lie *before* the maturation reaction with only the active state being able to mature. Furthermore, the physical situation may closer resemble a steady state where active protein is removed from the equilibrium by irreversible maturation, possibly in a chaperone dependent fashion [16], and inactive protein is removed, e.g. by protease degradation or aggregation. In this scenario, maturation kinetics could also influence the apparent stabilities and thus the outcome of GMMA. Here, we assume that the simple equilibrium Eq. 1. approximates the situation well and keep in mind that the absolute stabilities estimated here should not be compared directly to denaturation experiments of mature GFP.

Model estimation

The global fit of the effects of the individual substitutions is complex for at least two reasons. First, the global parameters including baselines (Fig. 1a, black line) are estimated simultaneously with all individual variant stabilities (Fig. 1a, abscissa values). The trade-off between adjusting the curve or the points may result in a rough optimization surface featuring many local optima. Second, many substitutions are observed in only few variants and may be poorly determined with greater

uncertainties that are combined with otherwise well-determined substitution effects. To address these complexities, we use three-step estimation procedure that relies on initial estimates of individual stability effects (Fig. 1d). The first two steps focus on achieving initial estimates of global parameters (Fig. 1e) and individual stability effects (Fig. 1f) using a mean-stability approach. The final global optimization in step three enhance consistency by allowing the fit to converge to the nearest optimum (Fig. 1g). The procedure builds on the biophysical interpretation of GMMA and thus, we describe it in some detail here.

In the first step, an initial global wild-type stability, ΔG_{wt}, is estimated together with the average effect of a substitution, $\langle \Delta\Delta G \rangle$ (Fig. 1e). This average effect replaces the sum in Eq. 2:

$$\Delta G_v \approx \Delta G_{\mathrm{wt}} + N_v \langle \Delta\Delta G \rangle \tag{3}$$

where, N_v is the number of substitutions in variant v. While this assumption is rather crude, it is only used in the estimates of initial values in step two, and it becomes robust with an increasing number of variants of each N-mutant (i.e. a variant with N substitutions). The average stability effect, $\langle \Delta\Delta G \rangle$, may be biased by IFS (which are in principle infinite) and these are therefore not used in the fit of ΔG_{wt} and $\langle \Delta\Delta G \rangle$ in steps one and two (see Methods).

In the second step, stability effects of individual substitutions are estimated by an approach similar to that used in the first step (Fig. 1f). For each substitution, s, $\Delta\Delta G_s$ is estimated from the subset of variants that contains that substitution. The effect is included in Eq. 3 by replacing one term of the average stability with the specific stability effect of s to be estimated, with the remainder of the terms described by an average effect:

$$\Delta G_v \approx \Delta G_{\mathrm{wt}} + \Delta\Delta G_s + (N_v - 1)\langle \Delta\Delta G \rangle \tag{4}$$

A robust fit to Eq. 4 requires a sufficient number of N-mutants (to estimate the average brightness) for several different values of N, but in contrast to above we only consider the subset of variants that contains the substitution s. To illustrate the required number of variants per substitution, figure 1f shows three examples of fits to Eq. 4 where it is clear that the 14 observed variants containing L178V result in average values of the N-mutant brightness that do not fit the model well. In contrast, the stabilizing V163A and destabilizing C48S fit the model well with 677 and 194 observed variants, respectively.

When all initial stability effects have been estimated by this mean-stability approach, we perform a global optimization in step three using damped least-squares optimization from which uncertainties are calculated (see Methods).

The three-step estimation strategy ensures that effects that are poorly estimated due to few observations, typically < 10-20 depending on the distribution on N-mutants (supplementary Fig. S1), do not affect the initial estimation for substitutions with good data. A 5-fold cross validation shows that the model is able to predict the brightness of GFP variants not used in training accurately with an RMSD on brightness of 0.25 (ca. 10% of the dynamic range from 1.4 to 3.8) and a Spearman's correlation coefficient r_s=0.87 (Fig. 2). Notably, the most substituted variants are predicted well with 73% and 91% of active variants (brightness >2.7) correctly predicted as active for variants with nine or more substitution and variants with between six and eight substitutions respectively.

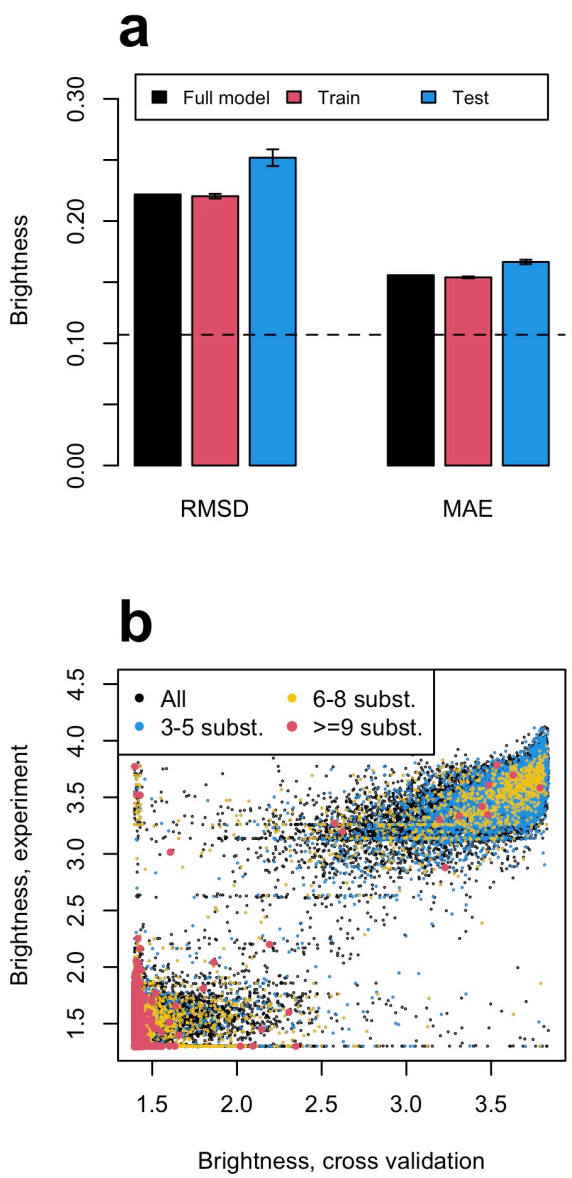

Figure 2. Five-fold cross validation of GFP multi-mutants where each variant was randomly assigned to one of five groups. (a) Root mean square deviation (RMSD) and mean absolute error (MAE) of predicted brightness for the full model fitted to all data (black) and the mean of the training and test data of the five cross validations (red and blue respectively). The error bars show the standard deviation from the five models each trained on ~80% of data and the dashed line indicates the experimental standard deviation of the wild-type brightness. (b) Predicted vs. experimental brightness of all variants, where the prediction of each variant is from the cross-validation when used as test data. The variants are colored according to the number of substitutions to show that even the mostly mutated variants are well predicted.

We estimate the stability of the "wild-type" sequence (avGFP + F64L) to be -2.8 kcal/mol. This is substantially smaller in magnitude than values < -10 kcal/mol reported from unfolding experiments of αGFP (avGFP + Q80R:F99S:M153T:V163A); however, unambiguously determining the stability of GFP is challenged by at least one folding intermediate of stability -3.7 kcal/mol [17]. The discrepancy may also be explained by *in vitro* unfolding experiments being qualitatively different from the equilibrium probed by GMMA. As discussed above, GMMA probes the stability of the pre-mature GFP fold and its ability to mature irreversibly in the context of a cell, whereas unfolding experiments

241 deactivates the mature protein *in vitro*. Since maturation is spontaneous, the pre-mature structure
242 is likely less stable compared to the mature protein and thus, GMMA could probe a less stable
243 structure compared to unfolding experiments of the mature protein.

244 *GFP substitution effects*

245 Using GMMA we obtain accurate estimates for 1,107 substitution effects (59% of the 1,879 present
246 in the library) with 80% found to be destabilizing and 8% stabilizing (Fig. 3). In the following we
247 highlight some points that can be learned about GFP from these.

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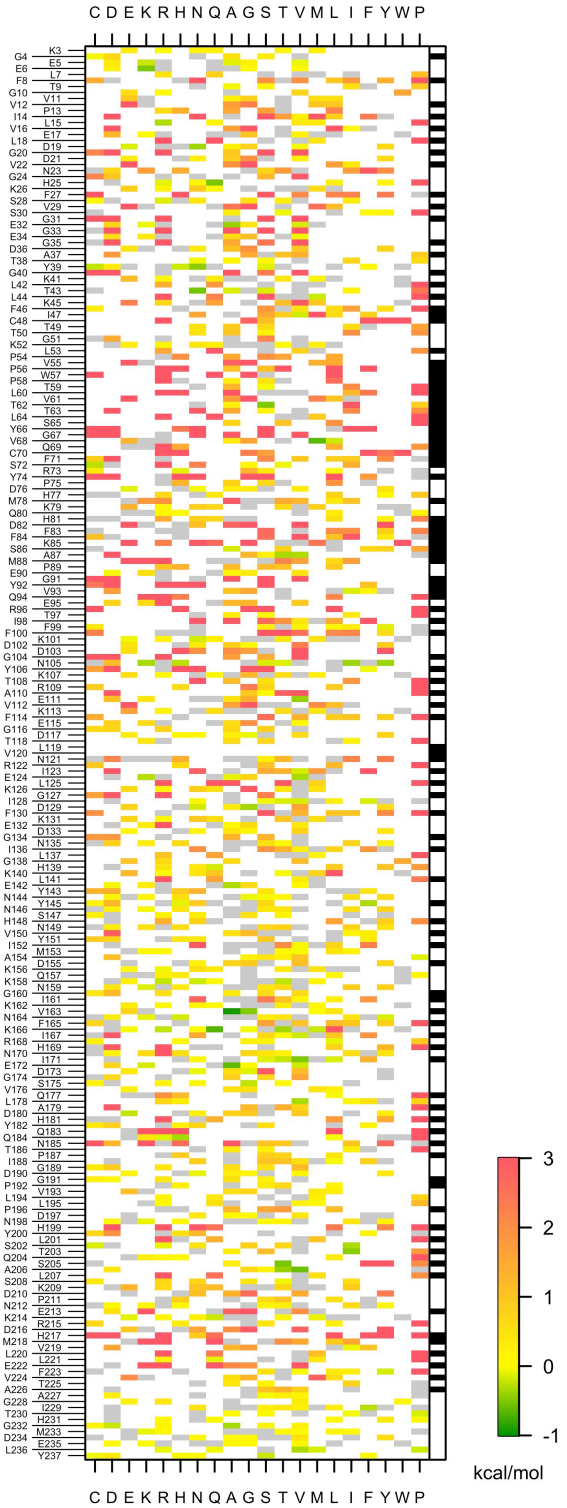


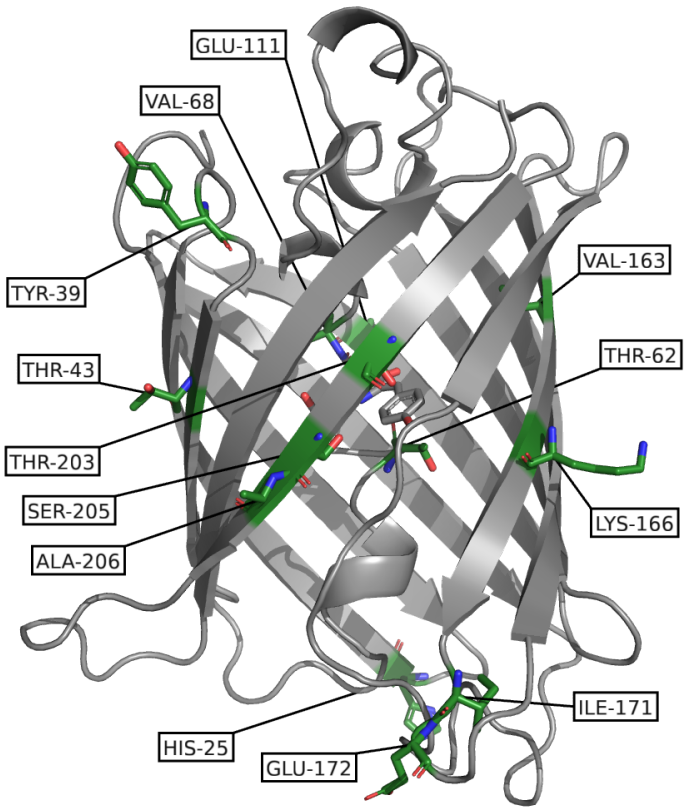
Figure 3. Heatmap showing the 1107 single-substitution effects estimated by GMMA from the multi-mutant library of GFP. Green indicates a stabilizing substitution, yellow are substitutions with close-to-zero effect, and orange to red indicate levels of destabilizing substitutions. Substitutions in gray are observed in the library but poorly estimated and white substitutions are not observed in the data. The rightmost column shows the solvent exposed residues in white and buried residues in black.

The majority of stabilizing substitutions are found at solvent exposed positions (63/83; 76%; Fig. 3) together with almost all substitutions with insignificant effect, i.e. $\Delta\Delta G_s$ close to zero (126/140; 90%). Both are enrichments compared to the overall fraction of exposed substitutions (712/1,107; 64%). Substitutions at buried positions are almost always destabilizing (361/395; 91%) with the notable exception of the top-ranking substitution, V163A, which is estimated to stabilize -0.94 ± 0.04 kcal/mol which is substantially more than the second best substitution (K166Q -0.68 ± 0.17 kcal/mol) and has low uncertainty due to a large amount of observed variant contexts in the data (supplementary Fig. S2). Another indication that V163A is highly beneficial is that another substitution at this position, V163G, is found to be the 12th most stabilizing substitution.

A group of positions with enrichment of stabilizing substitutions is solvent exposed Phe, Ile and Leu for which 11/64 (17%) are stabilizing. With the exception of two Ile to Phe substitutions, these tend to slightly decrease hydrophobicity which is in apparent contrast to other engineering studies that find increased surface hydrophobicity to be stabilizing [18,19] but fits well with the observation that such stabilizations tend to have poor additive contributions [19].

Surface positions with wild-type Glu have a significantly higher fraction of stabilizing substitutions, 12/71 (17%), as compared to Asp with only 5/90 (6%) substitutions being stabilizing. This may suggest a qualitative difference in the role of these two negatively charged amino acids (that otherwise have the same transitions in the codon table) in the context of a beta-barrel. Furthermore, four of the 15 most stabilizing substitutions remove a Glu sidechain from the surface (Fig. 4).

a



b

Top 1-3	**V163A**, K166Q, V68M
Top 4-6	E172A, **A206V**, T43N
Top 7-9	H25Q, S205T, E6K
Top 10-12	T62S, **I171V**, T203I
Top 13-15	**Y39N**, E111V, E32A

Figure 4. Positions (a) of the top 15 stabilizing substitutions (b) shown on the structure of GFP (PDB entry 1EMM). Position 163 has two substitutions within top 15 and rank 9 is not resolved in this structure. Substitutions known from sfGFP are marked in bold in (b).

All 42 substitutions at all ten Pro residues in GFP are found to be destabilizing, which is not surprising considering the structural role of Pro. Other positions that only show highly destabilizing substitutions include the chromophore positions Y66 and G67 and the maturation-related sites R96 and E222 [20].

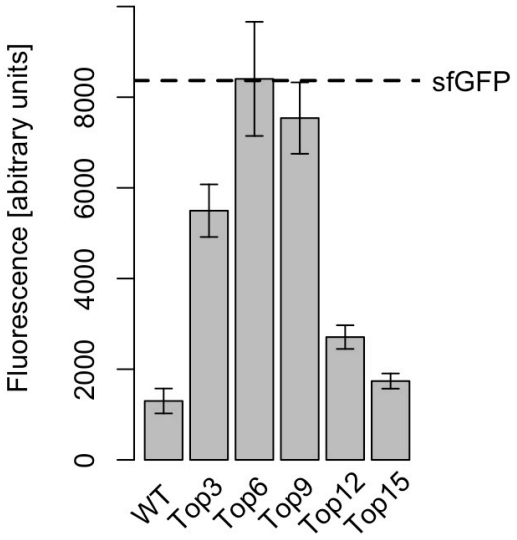
To examine whether the variant effects obtained from GMMA could be rationalized by structural stability of the mature state of GFP, we used Rosetta to predict the change in thermodynamic stability for all individual substitutions. Overall, we find a relatively good correlation between GMMA and Rosetta (r_s=0.6; supplementary Fig S3a). However, when we focus on those substitutions identified by GMMA as most beneficial we find that most are not identified by the structural stability calculations since none of the top 15 substitutions overlap (supplementary Fig S3a). We further find that the Rosetta calculations correlate more strongly with the results of GMMA than of the measured brightness of singly-substituted variants (r_s=0.6 vs r_s=0.4; Fig. S3), lending further support to the accuracy of GMMA.

GFP engineering based on GMMA

Having used GMMA to identify individual substitutions that can stabilize GFP and thereby suppress the detrimental effect of other substitutions, we examined whether these substitutions alone could be combined to improve GFP. Thus, we progressively inserted the 15 most stabilizing substitutions

296 into GFP in sets of three (Fig. 4b) and measured fluorescence levels during expression in *E. coli* at 41
297 °C. Measuring fluorescence directly in cells was intended to resemble the conditions of the assay
298 used to generate the data [6] while the elevated temperature was applied to enhance the associated
299 differences in thermal stability. As references we compare to the "wild-type" and sfGFP.

300 We find that all variants show fluorescence levels that are higher than the reference sequence (Fig.
301 5 and supplementary Fig. S4) with the Top6 variant giving as high an in-cell signal as sfGFP. Notably,
302 Top6 and sfGFP only have two substitutions in common, V163A and A206V, (Table 1 and
303 supplementary Table S1) whereas sfGFP contains 10 substitutions relative to the "wild-type"
304 (excluding S65T). While the Top12 and Top15 variants have lower fluorescence than Top6 and 9
305 they are still at least as bright as the reference wild type. We expect the increased fluorescence
306 mainly to reflect a difference in protein levels (i.e. not increased intrinsic fluorescence) and thus a
307 proxy for stability.



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309 **Figure 5.** Experimental test of multiply-substituted GFP variants composed of top ranking substitutions identified
310 by GMMA. Error bars show the standard deviation between four replicates.

311 These experiments demonstrate two important points of GMMA. First, that at least the six top-
312 ranking substitutions appear to have a substantial additive positive effect, with Top6 giving
313 significantly more signal than Top 3. One might suggest that the leveling off of Top9 and the
314 decreased signal of Top12 and Top15 relative to Top6 be caused by context-specific effects, due e.g.
315 to the close proximity between I171V and E172A or T203I, S205T and A206V (Fig. 4). The latter
316 combination, however, appears to be close to optimal in other high-throughput experiments [21].
317 Second, none of the 15 most highly ranking substitutions are highly deleterious (all variants are fully
318 functional). This observation supports the robustness of the outcome of GMMA since these
319 stabilizing substitutions have all been observed in a diverse set of multi-mutants in which they have
320 been judged to play a stabilizing role.

321 In addition to the calculated uncertainties, GMMA offers transparent analysis of the final fits of
322 individual substitutions (supplementary Fig. S2). These reveal that effects of the top 5 ranking
323 substitutions are well-determined by the data, whereas rank 6 (T43N) is only observed in nine

variants and thus we have less confidence in this. While V163A and A206V are both present in sfGFP, the high fluorescence of Top6 suggests that K166Q, E172A and V68M may be promising substitutions for GFP engineering. Continued experimental investigation of these specific effects is interesting, but beyond the scope of the present study.

Known GFP substitutions

To examine whether the substitutions we identify using GMMA more generally have stabilizing and background-insensitive effects, we compare these to substitutions that are known to be beneficial for GFP in multiply substituted contexts found in the literature [15,16,22—25] (see Methods). Known substitutions are in general found to be stabilizing or with insignificant effect (Fig. 6a) and GMMA outperforms both Rosetta stability calculations and experimental measurements of single-substitution effects in the identification of known substitutions (Fig. 6b). Known substitutions that are estimated by GMMA to be destabilizing (supplementary Table S1) may indeed also be accurate because they were originally selected for other purposes than stability. Perhaps most notable are the chromophore substitution S65T, selected for spectral properties, and C48S, introduced to avoid cysteine oxidation in extracellular environments. Likewise, the substitutions Q80R and H231L, here estimated to be slightly destabilizing, are historical substitutions caused by early PCR errors which are still present in some synthetic genes of GFP variants [26].

We furthermore consider 136 substitutions found in GFP variants deposited to the PDB. As expected, these are found to be stabilizing or with insignificant effect except for four substitutions found to be slightly destabilizing (less than half of the wild-type stability). In total, we find that 19 of the top 30 stabilizing substitutions obtained from GMMA are known and that none of the known substitutions are estimated to inactivate GFP ($\Delta\Delta G > \Delta G_{wt}$).

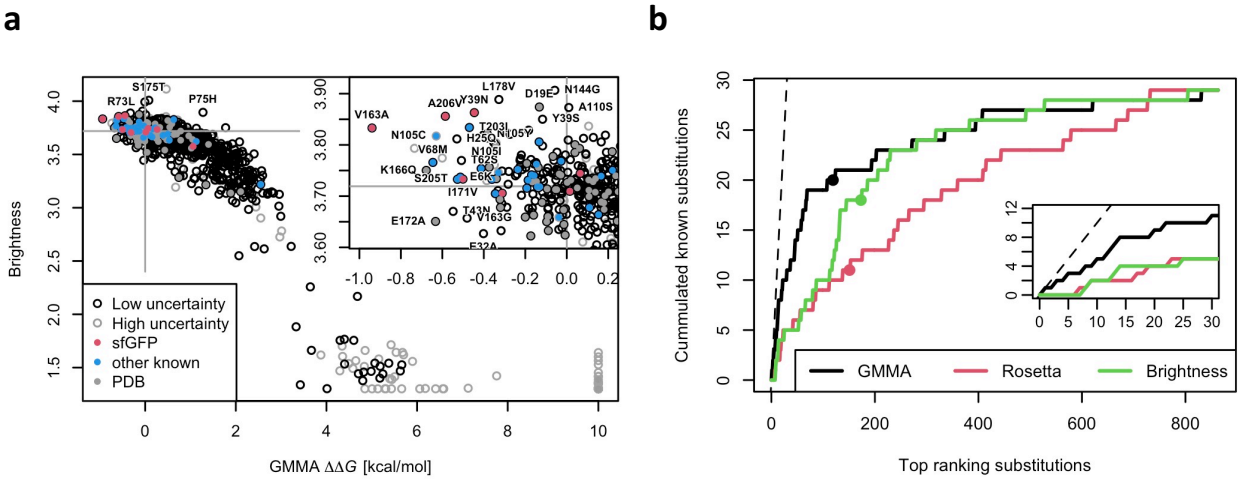

Figure 6. a) Brightness versus GMMA stabilities for single amino acid variants. GMMA estimates with large uncertainties are shown in grey circles. Substitutions known from optimized versions of GFP are shown in color. The vertical lines mark $\Delta\Delta G$=0 and the horizontal lines the brightness of the wild-type sequence. The insert shows the highly stabilizing, high brightness region and is enriched in known GFP variants. b) The graph shows the number of known substitutions within a given top-ranking number of substitutions according to GMMA (black), Rosetta (red) or singly-substituted variant fluorescence measured in the original data (green). The plot considers 29 unique known substitutions among the 863 low uncertainty substitutions for which single-variant fluorescence measurements and Rosetta calculations were available (see methods). For GMMA and Rosetta, the points mark $\Delta\Delta G$=0 and for brightness, the point marks the wild-type brightness (gray lines in panel a). The dashed line indicates maximum performance, i.e. the 29 known substitutions as the top ranking 29 substitutions.

The stability effects estimated by GMMA correlate strongly with the observed brightness of singly-substituted variants (Fig. 6a; r_s=-0.77). This, however, is mainly caused by the fact that the averaged FACS readout and GMMA analysis agree on destabilizing/low brightness variants. In contrast, when the goal is the more challenging one of identifying stabilizing variants we see a much weaker correlation between the observed brightness and GMMA (r_s=-0.26 for variants identified by GMMA as significantly stabilizing; see Methods) because individual stabilizing variants have only modest effects on brightness when introduced in an already stable background (Fig. 6a, insert).

Conclusions

We have here introduced a concept to quantify stability effects of individual substitutions through a global analysis of high-throughput assay measurements of multi-mutants. We demonstrate that the effect of single amino acid substitutions may be better determined by analysis of multi-mutants and have presented a Global Multi-Mutant Analysis (GMMA) that implements this. Since the analysis is based on a functional read-out, the identification of stabilizing substitutions is carried out while considering the assayed function of the protein and thus excludes structural stabilization that does not support the assayed function. Our cross-validation analysis demonstrates that GMMA can be used to predict experimental results on a complex fitness landscape involving nine or more substitutions in many variants which is also confirmed by fluorescence measurements of multiply-substituted GFP variant that contains the top ranking GMMA substitutions. We show that the six best substitutions may be combined to design a GFP variant which, under the assay conditions, is highly fluorescent and identify at least three enhancing substitutions, K166Q, V68M and E172A, that are known but seems underestimated in the literature of enhanced GFP variants. Finally, we show that GMMA is able to identify a large number of the substitutions that are known to be beneficial to GFP in multiple-substituted contexts.

Data and code availability

The code and dataset generated and analyzed during the current study are available in the GitHub repository, https://github.com/KULL-Centre/papers/tree/master/2020/multi-mutant-analysis-Johansson-et-al.

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Contributions

KEJ, KLL and JRW conceptualized the method. KEJ, KLL and JRW developed the method and analyzed the data. KEJ implemented the software and wrote the manuscript with contributions from all authors.

Methods

We assume that the observed activity, *F*, of a variant, *v*, is proportional to the fraction of the protein found in the active state A:

$$F \propto f_v = \frac{[\mathrm{A}]}{[\mathrm{A}] + [\mathrm{D}]} = \frac{1}{1 + \exp[\Delta G_v / RT]} \tag{5}$$

where [A] and [D] are the concentrations of active and inactive protein respectively, *R* is the gas constant and *T* the temperature. Following the original report of the data [6], the log fluorescence, or brightness *F*, is assumed proportional to the fraction of active protein. We carried out all data analyses using the *R project for statistical computing* with packages *minpack.lm* and *igraph.*

Initial estimation

The initial estimation of stability effects considers the *average* effect of substitutions, $\langle \Delta\Delta G \rangle$, which is sensitive to highly destabilizing substitutions. Thus, we first detect *irreversible fatal substitutions* (IFS) that are inactive in all contexts and have an unlikely pattern of activity among multi-mutants, e.g. many inactive double mutants in the case of GFP (see supplementary Appendix 2). We stress that the IFS are detected directly from an analysis of the data and is not based on any pre-defined notions of variants or regions that are particularly relevant for function. Variants containing

479 nonsense mutations are also generally expected to be IFS. We exclude 62 nonsense IFS and 51
480 missense IFS from the initial estimation together with the 2,310+4,851 variants that contain these
481 nonsense and missense IFS substitutions respectively.

482 The wild-type stability, ΔG_{wt}, is first estimated together with the average effect of all substitutions,
483 $\langle \Delta\Delta G \rangle$, using Eq. 6 which is simply a combination of Eqs. 3 and 5. These are fitted to the average
484 brightness of the *N*-mutants, $\langle F \rangle_N$, i.e. the average brightness of double-mutants, triple-mutants,
485 etc.

$$\langle F \rangle_N = \frac{\alpha_{\mathrm{A}} + \alpha_{\mathrm{D}} \exp[-(\Delta G_{\mathrm{wt}} + N\langle \Delta\Delta G \rangle)/RT]}{1 + \exp[-(\Delta G_{\mathrm{wt}} + N\langle \Delta\Delta G \rangle)/RT]} \tag{6}$$

486 Here, a constant baseline, α_{D}, for the brightness of the inactive variants is fitted whereas a constant
487 baseline for active variants, α_{A}, is not fitted independently but calculated from ΔG_{wt} and the
488 brightness of the wild-type sequence, F_{wt}, during fitting:

$$\alpha_{\mathrm{A}} = F_{\mathrm{wt}} + (F_{\mathrm{wt}} - \alpha_{\mathrm{D}}) \exp[-\Delta G_{\mathrm{wt}}/RT] \tag{7}$$

489 This makes α_{A} less sensitive to noise and outliers in the variant readout by relying on the data point
490 (ΔG_{wt}, F_{wt}) which is experimentally well-determined with 3,645 barcodes in the high-throughput
491 assay, i.e. individual observations of wild-type nucleotide sequence (2,444) or synonymous
492 sequences [6]. A standard error of each parameter is calculated as the square root of the diagonal
493 of the inverse Hessian matrix. The initial wild-type stability is estimated to $\Delta G_{\mathrm{wt}} = -1.8 \pm 0.2$
494 kcal/mol, the average effect of substitutions $\langle \Delta\Delta G \rangle = 0.45 \pm 0.04$ kcal/mol, and $\alpha_{\mathrm{D}} = 1.43 \pm$
495 0.03 (Fig. 1e). This results in $\alpha_{\mathrm{A}} = 3.8$.

496 In step 2, we use the values of ΔG_{wt}, $\langle \Delta\Delta G \rangle$ and the baseline parameters from step 1, to estimate
497 initial values of the individual substitutions, $\Delta\Delta G_s$, from the subset of variants that contains the
498 substitution, *s*, using Eqs. 4 and 6 (Fig. 1f). The use of a fixed value of $\langle \Delta\Delta G \rangle$ makes the initial
499 estimates robust and self-consistent for the global fit, and the approach is further supported by the
500 observation that variant effects are mostly independent of the details of the background [13]. Since
501 we always only change the background with a single substitution, $\langle \Delta\Delta G \rangle$ is not expected to vary
502 much and in practice less than the uncertainty that results from small sets of variants. We require
503 that all subsets have a diversity of at least 3 different multi-mutants that spans the transition region
504 and gives a fit with standard error < 1.0 and an absolute deviation < 5.0 log fluorescence units. With
505 this, we find that 56% of substitutions have sufficient data for this initial estimation. We then
506 afterwards estimate the remaining initial $\Delta\Delta G_s$ values from these well-determined effects.

507 *Graph analysis*

508 We pre-process the data for the global multi-mutant analysis, and build a bipartite graph by
509 assigning protein variants to one layer of nodes and another layer of individual substitutions. All
510 variant nodes are linked to the substitution nodes that the variant is composed of. We check that
511 all nodes are connected in the graph. If a subset of variants is composed of a subset of substitutions
512 that does not occur in the rest of the variants, this graph becomes disconnected from the rest, and
513 GMMA may be carried out on the subset alone. Since a single-mutant library is fully disconnected,
514 GMMA cannot be applied to this. Single mutants do not inform the global fit more than any other
515 variants and indeed 130 stability effects are estimated without the single mutant being observed.
516 Substitution nodes with a single link represents substitutions that contributes one parameter and
517 only one data point to the global analysis, referred to as hanging nodes. These do not inform the

global optimization and, thus, the effect of these are calculated after the global optimization. In summary, the graph is cleaned for 7 disconnected node pairs, and 257 hanging substitution nodes together with 255 dependent variants nodes. Finally, the graph is examined to ensure that no pair of substitutions only occurs together as these would make them impossible to distinguish and should be reparametrized as a single effect. The graph analysis is independent of the initial estimates and considers all data, including IFS and non-sense substitutions.

Global estimation

With input from both the initial estimation and the graph analysis, we perform the global optimization using the same damped non-linear least squares algorithm as above. We optimize all stability effects $\{\Delta\Delta G_s\}$ and the wild-type stability ΔG_{wt} to the nearest optimum (Fig. 1g). The baselines are fixed at the values determined in the initial fit (1.4 and 3.8). The stability effects are limited to the range -5 to 10 kcal/mol for robustness. With analytically calculated gradients, the global optimization of 1,616 parameters from 53,763 data points took 4-5 hours on a normal laptop.

The fit has a reduced chi-square $\tilde{\chi}^2 = 4.4$ which indicates that some parts of the data do not fit the model; however, we note that the cross-validation analysis shows that the data is robustly fitted and with at most a small level of overfitting (Fig. 2). Our fit appears notably better than that reported previously for similar models [6, 8] which we expect to be related to our inclusion of the global parameter, ΔG_{wt}. One contribution to an elevated $\tilde{\chi}^2$ could be the use of the distribution of the observed brightness of the wild-type sequence as a proxy for the variation of all variants, which may indeed have higher uncertainties in the assay.

Uncertainties

In the global analysis, we estimate the uncertainties from the covariations in the inverted Hessian matrix. We calculate two error measures to judge the uncertainty and reliability of the stability effects. The first, δ_s, is calculated using the log-fluorescence measurement uncertainty, reported to be $\delta F_{\text{wt}} = 0.11$ for the wild-type sequence [6], propagated via the covariance matrix diagonal and multiplied by 3 to get the 99.7% percentile (and to somewhat compensate for the expectation that the variants have higher uncertainty than the wild type):

$$\delta_s = 3 \left| \frac{\partial \Delta\Delta G_s}{\partial F} \right| \delta F_{\text{wt}} \tag{8}$$

A resampling experiment suggests that this measure captures the estimation accuracy well and we report this uncertainty as the stability error with ± notation (supplementary Fig. S1).

The second uncertainty, $\delta_{\Delta\Delta Gs}$, is used to filter out unreliable stability effects and is calculated from:

$$\delta_{\Delta\Delta Gs}{}^2 = \left(\frac{\partial \Delta\Delta G_s}{\partial F} \right)^2 \frac{\text{RSS}_s}{\text{DOF}_s} \frac{1}{V_s} \tag{9}$$

Again, the derivative is from the diagonal of the covariance matrix, RSS_s is the residual sum-of-squares of the variants used to estimate substitution *s*, DOF_s is the number of degrees-of-freedom and V_s is the number of variants used to estimate substitution *s*. The last factor gives an error-of-the-mean type of uncertainty that compensates for the case where a lucky fit of few variants is penalized. The number of degrees of freedom for a substitution assumes that a uniform fraction of parameters is estimated together with $\Delta\Delta G_s$ from the V_s data points

$$\text{DOF}_s = V_s\left(1 - {}^{S}/_{V}\right) - 2$$

where S and V are the total number of substitutions and variants respectively. We set a relatively conservative threshold and mark 772 (61%) stability effects with $\delta_{\Delta\Delta Gs} > 0.05$ kcal/mol as unreliably (a value that can be compared to $\langle \Delta\Delta G \rangle \approx 0.5$ kcal/mol). This low threshold has been set by manual inspection of plots that show the fit of each substitution to its respective subset of variants (similar to Fig. 1f). We use a hard threshold here to facilitate a clear discussion. However, in a specific application, substitutions could be judged individually based on both uncertainty measures and such plots, since many of the 772 poorly estimated substitutions are still informative. Notably, 222 (12% of all substitutions) are exclusively or predominantly observed in inactive variants and are therefore only represented in the flat region of the model. Thus, all of these may reliably be identified as destabilizing or even well determined on a range, even though the reported point estimate itself is highly uncertain. Of the remaining 550 (29%) substitutions with uncertain effects, the majority (398, 21%) are caused by poor statistics with five or fewer observed variants. The conservative threshold does exclude some substitutions with more than 10 observations (61 or 3%), that are potentially stabilizing, e.g. L221V, Q80K or E6A, and may be interesting depending on the application.

Classification

For the sake of discussion and early IFS identification, we classify all variants as either active or inactive. Variants with brightness below 2.7, half-way between maximum and minimum observed brightness in the original data, are assigned as inactive and the rest as active.

We mark substitutions with low uncertainty in the scores from GMMA (high/low uncertainty classification described above with the uncertainty calculation) as *significantly stabilizing* if the effect plus uncertainty is less than zero, *destabilizing* if the effects minus uncertainty is greater than zero and *insignificant* otherwise. Substitutions with high uncertainty are marked as destabilizing if the effect is more destabilizing than the wild-type stability and marked as unknown otherwise.

Solvent exposure categories are exposed or buried according to DSSP [27].

Rosetta stability effects

Rosetta $\Delta\Delta$G's were calculated according to protocols published in [28, 29] using precompiled Rosetta version 3.13 of June 2021 with the REF15 score function (ref2015_cart) and the chromophore parameters distributed with Rosetta (CRO.params). Calculations were based on PDB entry 1EMM that covers position Leu-5 to Ile-227 both included. This was prepared by 20 independent and unrestrained minimizations using the command:

```
./relax  -score:weights ref2015_cart  -relax:script cart2.script  -use_input_sc
-relax:cartesian  -ignore_unrecognized_res  -relax:min_type lbfgs_armijo_nonmonotone
-fa_max_dis 9.0  -extra_res_fa CRO.params
```

The lowest energy structure was used to evaluate all single variants using the command:

```
./cartesian_ddg -score:weights ref2015_cart  -ddg:iterations 3  -force_iterations false
-ddg::score_cutoff 1.0  -bbnbrs 1  -fa_max_dis 9.0  -ddg::cartesian  -ddg::legacy false
-extra_res_fa CRO.params
```

We used no special handling of substitutions involving proline and the substitution effect was calculated as the average of three repeats of the calculation. Results in Rosetta energy units (REU) were divided by 2.94 which have previously showed to correlate well with stability effects in

596 kcal/mol [28]. Nonsense and chromophore mutations could not be evaluated and calculations at
597 position Arg-80 were ignored because of the mismatch to our "wild-type" Gln-80.

598 *Known substitutions*

599 We focus on published variants that are relevant for our reference sequence (avGFP + F64L) and
600 consider the substitutions that constitute sfGFP [15], T-Sapphire GFP (tsGFP) [23], a split GFP
601 (splitGFP) [24], a computationally-optimized GFP known as des11 [16] and a tryptophan
602 chromophore variant called nowGFP [25] (supplementary Table S1). While many more variants exist
603 in the literature, these five variants represent close to all substitutions [22]. We expect substitutions
604 from these to be stabilizing or have an insignificant effect in GMMA. We note that some
605 substitutions from nowGFP have been reported to support the tryptophan chromophore [30, 31].

606 Additionally, we also consider 136 substitutions found in 147 structures of GFP, selected in the PDB
607 to have >90% identity to our wild-type sequence. Since these variants have all been expressed and
608 crystalized, we expect that these substitutions do not destabilize GFP substantially, i.e. lower
609 expectations compared to substitutions from the enhanced variants mentioned above.

610 *Experimental testing*

611 The chromophore substitution S65T is known to change the spectral properties of GFP and since
612 this is contained in sfGFP, we add this substitution to all variants (including the reference), in order
613 to compare fluorescence levels measured using the same excitation wavelength of 488 nm.

614 Synthetic genes encoding the multiply mutated GFP variants were custom synthesized by Twist
615 Bioscience in a pET29b expression vector and expressed using BL21(DE3) as host. The DNA sequence
616 was codon optimized for *E. coli* for the reference sequence, and only varied at the positions of
617 substitutions, where most common codons were used. Precultures were grown over-night and
618 normalized for cell density to approximately OD_{600} of 3.0, respectively. Expression was carried out
619 in 96-well plates which were inoculated with 5 μL in 45 μL LB medium containing kanamycin for
620 plasmid selection. After incubation at 37°C for two hours, IPTG was added to 1 mM and the
621 temperature was shifted to 41°C and fluorescence measured using a TECAN Infinite 200 Pro
622 microplate reader over the next 12 hours. All measurements were done with two independent
623 precultures, each with two technical replicates. The numbers reported in Fig. 5 are averaged over
624 the two last hours of the profiles in supplementary Fig. S4.