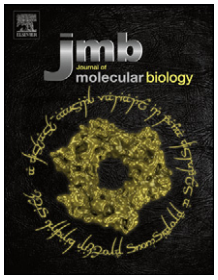

EASE-MM: Sequence-Based Prediction of Mutation-Induced Stability Changes with Feature-Based Multiple Models

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Abstract

Protein engineering and characterisation of non-synonymous single nucleotide variants (SNVs) require accurate prediction of protein stability changes ($\Delta\Delta G_u$) induced by single amino acid substitutions. Here, we have developed a new prediction method called *Evolutionary*, *Amino acid*, *and Structural Encodings with Multiple Models* (EASE-MM), which comprises five specialised support vector machine (SVM) models and makes the final prediction from a consensus of two models selected based on the predicted secondary structure and accessible surface area of the mutated residue. The new method is applicable to single-domain monomeric proteins and can predict $\Delta\Delta G_u$ with a protein sequence and mutation as the only inputs. EASE-MM yielded a Pearson correlation coefficient of 0.53–0.59 in 10-fold cross-validation and independent testing and was able to outperform other sequence-based methods. When compared to structure-based energy functions, EASE-MM achieved a comparable or better performance. The application to a large dataset of human germline non-synonymous SNVs showed that the disease-causing variants tend to be associated with larger magnitudes of $\Delta\Delta G_u$ predicted with EASE-MM. The EASE-MM web-server is available at http://sparks-lab.org/server/ease.

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Introduction

Accurate prediction of stability changes ($\Delta\Delta G_u$) induced by protein mutations is essential for successful protein engineering and characterisation of non-synonymous single nucleotide variants (SNVs) [1]. The most reliable methods for estimating $\Delta\Delta G_u$ employ the three-dimensional structure of a target protein and calculate the free energy difference before and after the mutation with an energy function [2,3,4,5,6,7]. The main disadvantage of this approach is that it cannot be used when the three-dimensional structure of the target protein is not available. Therefore, a number of machine learning methods emerged that can predict stability changes knowing the protein sequence only [8,9,10,11]. However, when independently evaluated, the performance of these methods is limited [12], especially for mutations in previously unseen non-homologous proteins [13]. Moreover, the accuracy varies for different types of mutations depending on the secondary structure (SS) or accessible surface area (ASA) of the mutation site [13].

Recently, we have reported that combining feature-based multiple models for the *two-state classification* of stability changes as stabilising or destabilising improves prediction accuracy and achieves more balanced results for different types of mutations [14]. In this work, we designed feature-based multiple models for the *real-value prediction* of $\Delta\Delta G_u$ and developed a publicly available web-server. Our method is called *Evolutionary*, *Amino acid*, *and Structural Encodings with Multiple Models* (EASE-MM). We compared the prediction performance of EASE-MM with related work in an extensive independent validation, in which we employed only proteins with $<25\%$ sequence identity to the dataset used for the design and training of our method. EASE-MM yielded improvements in both cross-validation and independent testing compared to other sequence-based

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methods. Moreover, EASE-MM, a sequence-based method, achieved a performance comparable to or better than structure-based energy functions. We applied our method to a large dataset of human germline non-synonymous SNVs and found that the disease-causing SNVs tend to be associated with larger magnitudes of $\Delta\Delta G_u$ predicted with EASE-MM. The significance of this finding is that EASE-MM, being a sequence-based method, can be applied to most single-domain monomeric proteins encoded in the human or other genomes.

Results

We have built EASE-MM, which comprises five specialised models to predict $\Delta\Delta G_u$ of mutations in residues located in different SS elements (helix, sheet, or coil) and with different levels of ASA (exposed or buried with a 25% threshold). The final prediction is the average of $\Delta\Delta G_u$ predicted with two models, one selected based on the predicted SS and the other based on the predicted ASA of the mutation site. We used a dataset of 1676 mutations (S1676) to design our method and estimate its performance using 10-fold cross-validation. Next, we employed two independent datasets of 543 and 236 mutations (S543 and S236, respectively) to confirm the robust performance of our method. Both datasets had a sequence identity $<25\%$ to the dataset used for the design and training of our method. Finally, we studied the relationship between disease-causing germline SNVs and $\Delta\Delta G_u$ predicted with EASE-MM.

Individual features

First, using the S1676 dataset, we examined the correlation between $\Delta\Delta G_u$ and each individual feature from a diverse set of 19 features encoding evolutionary conservation, amino acid parameters, and predicted structural properties of the mutation site (see Materials and Methods). The feature derived from the amino acid parameter bulkiness as the difference in the bulkiness of the mutant and wild-type amino acids (Δbulkiness) yielded the highest Pearson correlation coefficient (r) of 0.35. The best feature derived from evolutionary conservation was the difference of the position-specific scoring matrix (PSSM) probabilities of the mutant and wild-type amino acids (ΔPSSM) with an r of 0.27. The feature relative ASA (rASA) of the mutation site had the strongest correlation (r of 0.27) from all predicted structural properties. Supplementary Table S1 lists all individual features and their correlation coefficients for the S1676 dataset.

To illustrate the significance of combining features of different types, we evaluated every possible combination of two features. We used the support vector machine (SVM) [15] algorithm with a *linear* kernel function and 10-fold cross-validation to predict $\Delta\Delta G_u$ for the S1676 dataset. The top nine feature pairs were different amino acid parameters combined with either rASA or ΔPSSM with an r in the range of 0.42–0.46. Namely, the best two combinations were Δbulkiness + rASA and Δhydrophobicity + ΔPSSM. Supplementary Figure S1 shows $\Delta\Delta G_u$ as a function of Δbulkiness and rASA. The figure shows that the introduction of bulkier (relative to wild-type) amino acids in the protein core (low rASA) has a tendency to destabilise the protein structure.

Feature-based multiple models

Each of the five models employed by EASE-MM is specialised for a different type of mutations. To build these specialised models, we partitioned the training data (S1676) according to SS and ASA predicted from the protein sequence with the SPIDER method [16]. Then, a greedy sequential forward floating selection (SFFS) [17] algorithm was used to select the most relevant features for each data partition from the set of 19 features encoding evolutionary conservation, amino acid parameters, and predicted structural properties (see Materials and Methods). Fig. 1 depicts how EASE-MM predicts $\Delta\Delta G_u$. First, SS and ASA of the mutation site are predicted from the protein sequence. Then, one model is selected based on the predicted SS and one based on the predicted ASA of the mutation site. The final prediction is calculated as the average of $\Delta\Delta G_u$ predicted with the two selected models.

Supplementary Table S2 lists the selected features for each EASE-MM model, ranked by their contributions to the prediction performance. At least one of the two best performing amino acid parameters (Supplementary Table S1), Δbulkiness and Δhydrophobicity, was represented in each of the five models.

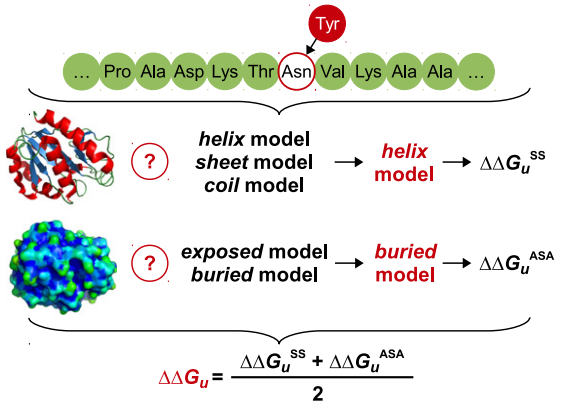

Fig. 1. EASE-MM calculates the stability change ($\Delta\Delta G_u$) as the average of $\Delta\Delta G_u$ predicted with two distinct models chosen based on the *predicted* secondary structure and accessible surface area of the mutation site.

Every model contained at least one predicted structural feature, the best structural feature, rASA, was included in all but one model. Also, at least one feature encoding evolutionary conservation was represented in all but one model. This highlights that combining features of different types is beneficial for predicting $\Delta\Delta G_u$. At the same time, each model comprised specific features not represented in other models, supporting our hypothesis of building multiple specialised models for different types of mutations. For instance, the amino acid attribute Δcompressibility was selected for the *helix* and *sheet* models but not for the *coil* model. Regarding the two ASA-based models, features Δisoelectric point and Δpolarisability were selected for the *buried* but not for the *exposed* model. To demonstrate that the five models of EASE-MM are indeed specialised, we permuted the labels of the five data partitions and observed a statistically significant decrease in prediction performance of 11–43% (Williams' test, $p < 0.01$, Supplementary Table S3).

Cross-validation performance

We conducted the first validation of our method using *unseen-protein* 10-fold cross-validation on the S1676 dataset (1676 mutations in 70 proteins). The *unseen-protein* 10-fold cross-validation ensures that any cluster of similar proteins is contained within a single cross-validation fold to prevent over-estimation of the prediction results (see Materials and Methods). We replicated the cross-validation 100 times with randomly re-generated folds and averaged the results. We compared the prediction performance of EASE-MM with our previous work, EASE-AA [13], which employs a single model trained using two evolutionary, three predicted structural, and seven amino-acid-based features. In the 10-fold cross-validation, EASE-MM yielded a Pearson correlation coefficient (r) of 0.56, which constitutes a relative improvement of 8% compared to EASE-AA with an r of 0.52 (Table 1). We performed

Table 1. Comparison of EASE-MM and related work in terms of correlation and error between the predicted and experimentally measured stability changes ($\Delta\Delta G_u$)

	All proteins				High-resolution crystal (≤3 Å) structures			
Method	Dataset	r[a]	p[a]	RMSE[a]	Dataset	r[a]	p[a]	RMSE[a]
EASE-AA	S1676[b]	0.52	4.2×10^{-5}	1.56	—	—	—	—
EASE-MM		0.56	—	1.52		—	—	—
I-Mutant2.0 *seq*[c]		0.32	4.8×10^{-11}	1.37		0.38	1.3×10^{-4}	1.34
MUpro 1.1		0.33	6.9×10^{-8}	1.32		0.37	4.6×10^{-4}	1.30
I-Mutant2.0 *str*[c]		0.36	1.8×10^{-8}	1.34		0.37	1.1×10^{-4}	1.34
Rosetta 3.5		0.38[h]	3.8×10^{-5}	3.58[hj]		0.35[h]	1.6×10^{-4}	4.00[hj]
FoldX 3	S543[d]	0.41	1.1×10^{-3}	1.87	S405[e]	0.42	0.027	1.92
DFIRE		0.45	8.4×10^{-4}	1.44		0.46	0.048	1.49
EASE-AA		0.48	2.0×10^{-3}	1.25		0.48	0.081	1.25
PoPMuSiC 2.1		0.53	0.909	1.21		0.49	0.404	1.27
EASE-MM		0.53	—	1.22		0.51	—	1.25
I-Mutant2.0 *seq*[c]		0.44	1.0×10^{-3}	1.18		0.43	0.032	1.08
MUpro 1.1		0.36	2.7×10^{-5}	1.20		0.29	3.9×10^{-4}	1.14
I-Mutant2.0 *str*[c]		0.52	0.105	1.07		0.47	0.160	0.94
Rosetta 3.5		0.27[i]	1.2×10^{-7}	1.88[ij]		0.34[i]	3.9×10^{-3}	1.94[ij]
FoldX 3	S236[f]	0.28	3.3×10^{-6}	1.70	S157[g]	0.34	9.6×10^{-3}	1.80
DFIRE		0.54	0.162	1.18		0.52	0.563	1.03
EASE-AA		0.53	0.025	1.10		0.51	0.172	1.04
PoPMuSiC 2.1		0.57	0.630	1.05		0.58	0.640	0.98
EASE-MM		0.59	—	1.03		0.55	—	0.97

[a] r, Pearson correlation coefficient; p, probability that the correlation coefficients (r) of the given method and EASE-MM are different due to random chance (Williams' test for comparing correlation coefficients); RMSE, root mean square error [kcal mol^{-1}].
[b] S1676 was used for feature selection and 10-fold cross-validation; the sequence identity of any two proteins from two different cross-validation folds was < 25%.
[c] *seq*, sequence-based; *str*, structure-based.
[d] S543 is an independent test set, not used for feature selection nor model optimisation, compiled from a subset of the dataset from Dehouck *et al.* [3] with a sequence identity < 25% to S1676, S236, and S157.
[e] S405 is a subset of S543 containing only mutations in high-resolution (≤3 Å) crystal structures.
[f] S236 is an independent test set, not used for feature selection nor model optimisation, with a sequence identity < 25% to S1676, S543, and S405.
[g] S157 is a subset of S236 containing only mutations in high-resolution (≤3 Å) crystal structures.
[h] Three mutations were removed due to atomic clashes ($E_{rep} > 7$).
[i] Four mutations were removed due to atomic clashes ($E_{rep} > 7$).
[j] $\Delta\Delta G_u$ predicted with Rosetta is in Rosetta energy units, no scaling was performed.

Williams' test for comparing correlation coefficients [18] to confirm that the improvement was statistically significant with $p \ll 0.01$. Supplementary Figure S2 shows the experimentally measured $\Delta\Delta G_u$ as a function of $\Delta\Delta G_u$ predicted with EASE-AA and EASE-MM for the S1676 dataset.

Using multiple models in this work was motivated by the fact that our previous work, EASE-AA, yielded an unbalanced prediction performance for different types of mutations. Fig. 2 shows the Pearson correlation coefficient (r) for different types of mutations based on SS and ASA. In this analysis, SS and ASA were calculated using DSSP [19] from experimentally determined structures. Regarding different SS types, EASE-MM was able to improve EASE-AA's performance for mutations located in α-helices with a relative improvement of 12% from r of 0.51 to 0.57 (Williams' test, $p \ll 0.01$). For β-sheets and coils, the improvements were not significant (r of 0.56 and 0.59, $p = 0.123$; r of 0.40 and 0.43, $p = 0.166$, respectively). Regarding buried (rASA $\leq$ 25%) and exposed (rASA $>$ 25%) mutation sites, both methods yielded a considerably lower correlation for the exposed residues. While EASE-AA yielded an r of only 0.31, EASE-MM achieved an r of 0.42, which represents a relative improvement of 35% (Williams' test, $p \ll 0.01$).

We also divided mutations based on the type of the wild-type and mutant amino acids (denoted as wild-type→mutant, Fig. 2). We considered mutations to alanine and mutations to any other amino acid type, mutations from small to large amino acids and *vice versa*, mutations from hydrophobic to hydrophilic amino acids and *vice versa*, and mutations from charged to polar amino acids and *vice versa*. EASE-MM achieved a statistically significant improvement for mutations to amino acid types other than alanine (Williams' test, $p \ll 0.01$) with a relative r improvement of 10% (r of 0.54) compared to EASE-AA (r of 0.49). This is interesting because many available datasets have a strong bias towards mutations to alanine. Particularly for S1676, 23% of mutations were any→alanine, whereas the second most common mutation, any→valine, represented only 7%. This result shows that EASE-MM is not biased (in fact, it has less bias relative to EASE-AA) towards any→alanine mutations despite their abundance in the training dataset. Regarding the other amino acid categories, EASE-MM achieved statistically significant improvements for large→small (Williams' test, $p \ll 0.01$) and charged→polar mutations ($p = 0.003$).

Independent test performance

We employed two different independent test sets, which were not used in any way for neither feature selection nor model optimisation, to verify if EASE-MM performs robustly. The S543 dataset comprised 543 mutations in 55 proteins with a sequence identity $<$ 25% to the S1676 training set. The second dataset (S236) contained 236 mutations in 23 proteins with a sequence identity $<$ 25% to S1676. The two test sets were disjoint (sequence identity $<$ 25%). We compared the performance of EASE-MM with three sequence-based methods (I-Mutant2.0 [8], MUpro [9], and EASE-AA [13]), four structure-based energy functions (Rosetta [5],

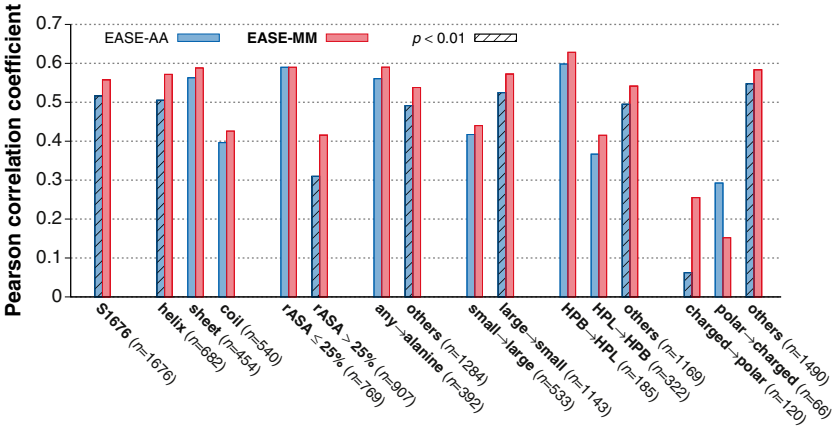

Fig. 2. Pearson correlation coefficient (r) as the performance of single-model EASE-AA [13] and multiple-models EASE-MM for different types of mutations from the S1676 dataset. The striped bars show results which are statistically different from EASE-MM (Williams' test, $p < 0.01$). The secondary structure elements (helix, sheet, coil) and relative accessible surface area (rASA) of the mutation site were calculated with DSSP [19]. We also divided mutations based on the type of the wild-type and mutant amino acids (denoted as wild-type→mutant). Small and large amino acids were defined based on the non-hydrogen atom counts. Amino acids were grouped based on their side-chains as hydrophobic (HPB): A, V, I, L, M, F, Y, W; polar: S, T, N, Q; charged: D, E, K, R, H; and hydrophilic (HPL): polar + charged.

FoldX [4], DFIRE [7], and PoPMuSiC [3]), and the structure-based version of I-Mutant2.0 [8]. Table 1 summarises the results in terms of r and root mean square error (RMSE). On the larger dataset (S543), EASE-MM yielded an r of 0.53 and was able to outperform all sequence-based as well as structure-based methods except for PoPMuSiC (Williams' test, $p<0.01$). The relative improvements were of 66% (sequence-based I-Mutant2.0), 61% (MUpro), 47% (structure-based I-Mutant2.0), 39% (Rosetta), 29% (FoldX), 18% (DFIRE), and 10% (EASE-AA). Fig. 3 shows the experimentally measured $\Delta\Delta G_u$ as

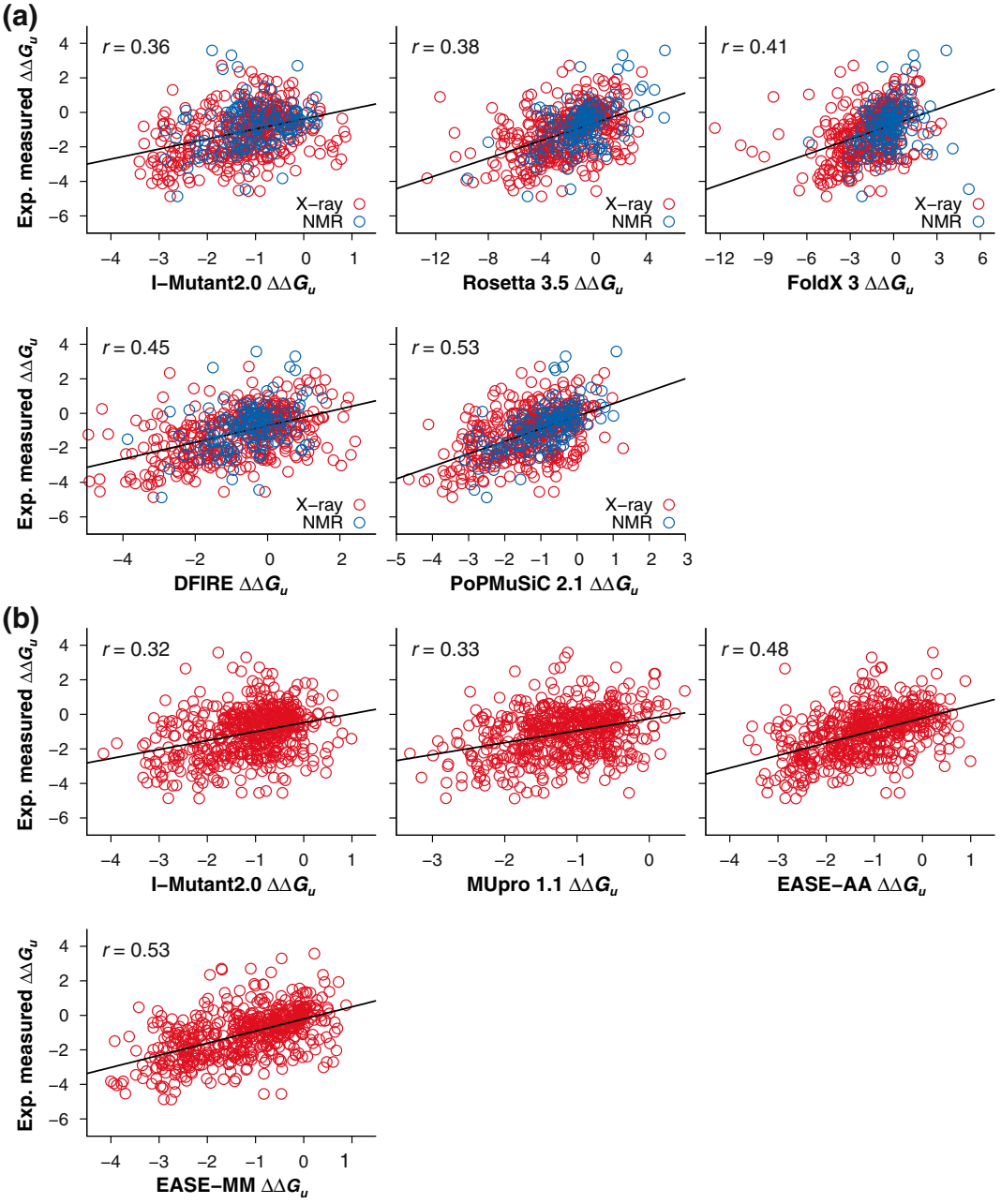

Fig. 3. Experimentally measured stability changes ($\Delta\Delta G_u$) from the S543 dataset as a function of $\Delta\Delta G_u$ predicted with the five *structure-based* methods **(a)** and four *sequence-based* methods **(b)** including EASE-MM. Three predictions which caused atomic clashes during structure optimisation with Rosetta ($E_{rep} > 7$) were removed from the Rosetta plot. For the structure-based methods **(a)**, X-ray denotes predictions for proteins with high-resolution (≤3 Å) crystal structures (405 mutations), and NMR denotes predictions for protein structures determined with nuclear magnetic resonance (138 mutations). The black lines are the linear regression fits.

a function of $\Delta\Delta G_u$ predicted with the nine compared methods for the S543 dataset. On the smaller dataset (S236), EASE-MM yielded an r of 0.59 and statistically significant improvements ($p<0.01$) compared to sequence-based I-Mutant2.0 and MUpro as well as structure-based Rosetta and FoldX. When compared to sequence-based EASE-AA (r of 0.53), the improvement was not significant ($p=0.025$). None of the nine compared methods was able to outperform EASE-MM. Supplementary Figure S3 shows the experimentally measured $\Delta\Delta G_u$ as a function of predicted $\Delta\Delta G_u$ for the S236 datasets.

To see if the performance of the structure-based energy functions was affected by the quality of the experimentally determined structures, we also evaluated the performance of all methods on subsets of S543 and S236 comprising only mutations in proteins with high-resolution ($\leq$3 Å) crystal structures. We refer to these datasets as S405 with 405 mutations in 44 proteins and S157 with 157 mutations in 16 proteins. While the performance of all methods changed marginally, no method was able to outperform EASE-MM at significance level $\alpha=0.01$ (Table 1).

Fig. 4 shows the prediction performance (r) of the three best-performing methods (EASE-AA, PoPMuSiC, and EASE-MM) on the S543 dataset for different types of mutations based on the SS and ASA of the mutation site and for different wild-type and mutant amino acid types. As it was expected, the most challenging mutations were located in coils, mutations to other amino acids than alanine, and mutations introducing a larger amino acid. Supplementary Figure S4 depicts the same comparison for all nine compared methods for both S543 and S236 datasets.

EASE-MM estimates the SS and ASA of the mutation site using SPIDER [16] from the protein sequence. To see if EASE-MM could make better $\Delta\Delta G_u$ predictions using experimentally determined structures, we substituted SPIDER predictions with the true SS and ASA assignments calculated with DSSP [19] from three-dimensional structures from the Protein Data Bank [20]. There was no statistical difference in the prediction performance (r) of $\Delta\Delta G_u$ for the two methods (Supplementary Table S4, Williams' test, $p=0.973$ and 0.446 for S543 and S236, respectively). As a control, we used randomly generated SS and ASA assignments and observed a significant decrease in the prediction performance from an r of 0.53 to 0.36 for S543 ($p\ll0.01$) and from 0.59 to 0.31 for S236 ($p=0.002$). These results demonstrate that SPIDER can reliably predict SS and ASA, and that their accurate prediction is important for reliable prediction of $\Delta\Delta G_u$ by EASE-MM.

Stability changes of disease-causing mutations

Finally, we investigated if the predicted stability changes could be used as an indication of disease-causing mutations. We compiled a dataset of 10,511 disease-causing non-synonymous SNVs in 2201 proteins from ClinVar [21] and 278,760 putatively neutral non-synonymous SNVs in 20,096 proteins from the 1000 Genomes Project [22]. Fig. 5 plots the distributions of the absolute value of $\Delta\Delta G_u$ predicted

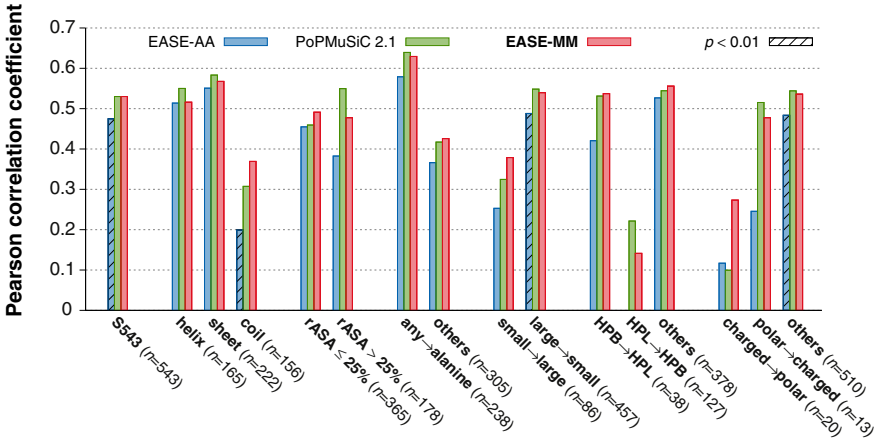

Fig. 4. Pearson correlation coefficient (r) as the performance of EASE-AA, PoPMuSiC, and EASE-MM for different types of mutations from the S543 dataset. The striped bars show results which are statistically different from EASE-MM (Williams' test, $p<0.01$). Some methods yielded a negative correlation, which is shown here as a missing bar. The secondary structure elements (helix, sheet, coil) and relative accessible surface area (rASA) of the mutation site were calculated with DSSP [19]. We also divided mutations based on the type of the wild-type and mutant amino acids (denoted as wild-type→mutant). Small and large amino acids were defined based on the non-hydrogen atom counts. Amino acids were grouped based on their side-chains as hydrophobic (HPB): A, V, I, L, M, F, Y, W; polar: S, T, N, Q; charged: D, E, K, R, H; and hydrophilic (HPL): polar + charged.

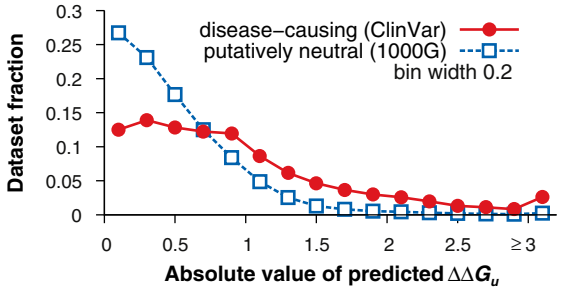

Fig. 5. Distributions of the absolute value of stability changes ($\Delta\Delta G_u$) predicted with EASE-MM for 10,511 disease-causing non-synonymous single nucleotide variants (SNVs) from ClinVar [21] and 50,910 putatively neutral non-synonymous SNVs from the 1000 Genomes Project (1000G) [22] with allele frequency (AF) $\geq$ 1%. The figure shows that neutral mutations prevail for small stability changes, while disease-causing mutations are characterised by more significant changes in the protein stability.

with EASE-MM for the 10,511 disease-causing and 50,910 putatively neutral mutations with allele frequency (AF) $\geq$ 1%. The two distributions were significantly different (Wilcoxon's test, $p \ll 0.01$). As expected, the figure shows that while putatively neutral mutations are characterised by small magnitudes of $\Delta\Delta G_u$, disease-causing mutations tend to have a larger absolute effect on the protein stability according to EASE-MM predictions. We employed the *absolute value* of $\Delta\Delta G_u$ because it has been shown that both protein destabilisation [1,23] as well as stabilisation, via loss of flexibility [24,25], can lead to a loss of protein function. That is, it is the magnitude rather than the direction of $\Delta\Delta G_u$ that might be indicative. The idea of using the absolute value of $\Delta\Delta G_u$ is further supported by a large-scale analysis of Casadio *et al.* [26]. There, the authors showed that the correlation of disease probability with the absolute value of experimentally measured $\Delta\Delta G_u$ was stronger than with $\Delta\Delta G_u$ of only the destabilising or stabilising mutations.

We also performed a binary classification of disease-causing and neutral SNVs using predicted $\Delta\Delta G_u$. Based on the receiver operating characteristic (ROC) curve analysis, EASE-MM yielded the area under the curve (AUC) of 0.69. We chose a prediction threshold of 0.8 kcal mol^{-1} based on the point where the distributions of disease-causing and neutral SNVs intersect in Fig. 5. This threshold resulted in a Matthews correlation coefficient (MCC) of 0.25 with a sensitivity (correctly predicted disease-causing SNVs) of 49% and specificity (correctly predicted neutral SNVs) of 80%, binary accuracy of 75%, precision (positive predictive value) of 33%, and negative predictive value of 88%. Thus, the statistically different distributions of predicted $\Delta\Delta G_u$ can provide some discriminative power between disease-causing and neutral SNVs. This means that $\Delta\Delta G_u$ predicted with EASE-MM can be useful in combination with other predictive features for improving classification of disease-causing SNVs.

Next, we investigated the relationship between the AF and $\Delta\Delta G_u$. The AF should generally reflect the fitness of the allele with respect to its intended biological function [27]. Hence, we hypothesised that protein stability could be one of the factors affecting the AF. Fig. 6 shows the absolute value of $\Delta\Delta G_u$ predicted with EASE-MM as a function of *binned* $\log_{10}$ AF for 278,760 non-synonymous SNVs from the 1000 Genomes Project [22]. There, AFs were grouped into bins so that each bin contained at least 500 SNVs. However, some bins were larger due to many SNVs with the same AF value. As expected, there was a strong negative correlation (r of –0.85) between the AF bins and the average of the absolute value of $\Delta\Delta G_u$. That is, highly populated alleles tend to have smaller changes in the protein stability.

Discussion

We have developed a machine learning method named EASE-MM, which can predict the change in the protein stability upon a single amino acid substitution based on sequence information, without the experimentally determined three-dimensional structure. EASE-MM employs multiple SVM models specifically designed for different types of mutations based on the SS and ASA of the mutation site. Each SVM model combines a different set of features encoding evolutionary conservation, amino acid parameters, and predicted structural properties. The new method yielded a robust performance with an r of 0.56 in 10-fold cross-validation and 0.53–0.59

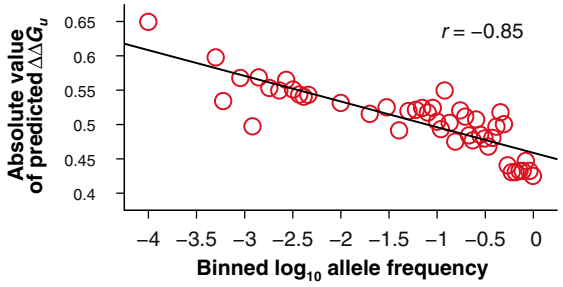

Fig. 6. Average of the absolute value of stability changes ($\Delta\Delta G_u$) predicted with EASE-MM as a function of binned $\log_{10}$ allele frequency (AF) for 278,760 non-synonymous single nucleotide variants (SNVs) from the 1000 Genomes Project [22], the average being calculated for bins containing at least 500 SNVs (some bins were larger due to many mutations with the same AF value). The black line is the linear regression fit. The figure shows a strong negative correlation demonstrating that highly populated alleles tend to have smaller changes in the protein stability.

in independent testing. EASE-MM outperformed other sequence-based methods and achieved a comparable or better performance than structure-based energy functions (Table 1).

To avoid over-training in a particular group of proteins, we coupled feature selection with the *unseen-protein* cross-validation procedure, in which we ensured that no two folds shared sequences with a pairwise identity $\geq 25\%$. A similar approach was used for our previous work [13,14] as well as for the prediction of disease-causing genetic variants [28,29,30]. Furthermore, we employed two different independent test sets to confirm that our method was not over-trained. These test sets had $< 25\%$ sequence identity to our training data to prevent over-estimation of prediction results [31].

Our work reveals several predictive features that are important for protein stability. For instance, sequence conservation is an expected feature because it has been long known to be a strong indicator of functionally and structurally important residues. Indeed, we found a positive correlation between $\Delta\Delta G_u$ and ΔPSSM of 0.27 ($p \ll 0.01$). Here, $\Delta\text{PSSM} = \text{PSSM}_{mt} - \text{PSSM}_{wt}$ and thus, the positive correlation means that an introduction of an uncommon amino acid type in a conserved position results in the destabilisation of the protein. Different features, however, contribute differently to different models. As shown in Supplementary Table S2, the most important features are rASA and changes in helix tendency for the *helix* model, sequence conservation and changes in amino acid volume for the *sheet* model, changes in hydrophobicity and flexibility for the *coil* model, changes in isoelectric point and bulkiness for the *buried* model, and changes in amino acid volume and predicted helix probability for the *exposed* model. These features were selected automatically using a feature selection algorithm to maximise the performance on the training dataset. Sometimes seemingly similar features were selected for distinct models. However, their interpretation might vary. For instance, on the one hand, the feature changes in helix tendency (Δhelix tendency) in the *helix* model suggests whether the preference to form helix is different for the mutant and wild-type amino acid types. On the other hand, the feature helix probability in the *exposed* model expresses the likelihood of the mutation site adopting a helical structure in the wild-type protein. One disadvantage of using machine learning models is that the selected features are not always easy to interpret. For example, also the *sheet* model contains the feature Δhelix tendency but its contribution in this model is not very significant.

We found that the new method not only outperformed our previous work, EASE-AA [13], but yielded a more balanced performance for different types of mutations based on the ASA of the mutation site (Fig. 2). That is, EASE-MM yielded improvements in correlation with experimentally measured $\Delta\Delta G_u$ for the exposed residues (rASA > 25%), which were more challenging to predict for both methods, while retaining the same performance as EASE-AA for the buried residues. Since EASE-AA uses a single model trained with the same types of features as implemented in EASE-MM, it means that the improved predictions can be mainly attributed to employing specialised models for different types of mutations.

While the new method can be applied universally to any amino acid sequence of a monomeric protein, it was trained and tested only using structured, soluble proteins. In fact, this is a common limitation to most prediction methods being a direct consequence of the available experimental $\Delta\Delta G_u$ data lacking membrane or intrinsically disordered proteins. Furthermore and particularly for EASE-MM, another limitation is that structural properties (SS and ASA) can be reliably predicted only for single-domain proteins. For the prediction of stability changes of protein–protein complexes, several structure-based methods are available [4,32]. Lastly, our method is built on experimental results that are sensitive to environmental variables such as pH, salts, and temperature. Although some of these environmental variables are recorded in the ProTherm database, the number of measurements at any given experimental condition is too small to include the environmental variables in our models or to estimate the achievable limits of the agreement between the computational predictions and experimental measurements.

Finally, we applied EASE-MM to a large dataset of human disease-causing and putatively neutral germline non-synonymous SNVs. We found that the distributions of predicted $\Delta\Delta G_u$ are significantly different (Fig. 5). Moreover, there was a strong negative correlation between the binned AF and average of the absolute value of predicted $\Delta\Delta G_u$ (Fig. 6). Our results show that highly populated alleles tend to be associated with smaller changes in protein stability. As pointed out elsewhere [33,34], the value of $\Delta\Delta G_u$ alone is not sufficient to provide reliable classification of SNVs because protein stability is only one of many disease-causing factors. Nevertheless, our results indicate that EASE-MM can be combined with other predictive features for improved characterisation of disease-causing mutations. The significance of this finding is that EASE-MM, being a sequence-based method, can be applied to most single-domain monomeric proteins encoded in the human or other genomes.

Materials and Methods

Datasets

We used several different datasets to design, validate, and independently test our method. Table 2 provides a summary of these datasets. We used the S1676 (1676

Table 2. Datasets used to design, validate, and independently test EASE-MM

Dataset	Mutation count						Protein count						Source
	all	H[a]	S[a]	C[a]	B[a]	E[a]	*all*	H[a]	S[a]	C[a]	B[a]	E[a]	
S1676[b]	1676	615	438	623	744	932	70	51	41	56	54	59	Folkman *et al.* [14]
S543[c]	543	155	224	164	292	251	55	32	33	39	44	42	Dehouck *et al.* [3]
S405[d]	405	87	195	123	231	174	44	25	27	31	34	35	subset of S543
S236[e]	236	81	62	93	109	127	23	12	12	17	18	16	Folkman *et al.* [14]
S157[f]	157	33	58	66	61	96	16	7	8	12	12	11	subset of S236
human	10,511 (disease) + 278,760[g] (neutral)						2201 (disease) + 20,096[g] (neutral)						ClinVar [21], 1000G [22]

[a] H, helix; S, sheet; C, coil; B, buried; E, exposed; the five SS and ASA partitions were predicted with SPIDER [16].
[b] training set and 10-fold cross-validation, the sequence identity of any two proteins from two different folds was < 25%.
[c] test set; S543 is independent to S1676, S236, and S157 with a sequence identity < 25%.
[d] test set; S405 is a subset of S543 containing only mutations in high-resolution (≤3 Å) crystal structures.
[e] test set; S236 is independent to S1676, S543, and S405 with a sequence identity < 25%.
[f] test set; S157 is a subset of S236 containing only mutations in high-resolution (≤3 Å) crystal structures.
[g] For the distribution analysis, a subset of 50,910 mutations (14,113 proteins) with allele frequency (AF) ≥ 1% was used.

mutations in 70 proteins) and S236 (236 mutations in 23 proteins) datasets compiled in Folkman *et al.* [14] from ProTherm [35] (version February 2013). ProTherm defines a stability change as the difference in the unfolding free energy: $\Delta\Delta G_u[\text{kcal mol}^{-1}] = \Delta G_u(mutant) - \Delta G_u(wild\text{-}type)$. Thus, destabilising mutations yield $\Delta\Delta G_u < 0$. We verified all records in ProTherm and corrected incorrect entries according to the original publications. Next, we removed all duplicate entries of the same amino acid substitutions (*e.g.*, different concentrations of chemicals). If several measurements of the same mutation under the same experimental conditions were present, we averaged $\Delta\Delta G_u$. If several measurements of the same mutation under different experimental conditions were present, we kept only the measurement closest to the physiological pH 7. The S1676 dataset was used to design our method and optimise all parameters. S1676 and S236 are mutually independent and do not share proteins with ≥ 25% sequence identity. Moreover, S236 is also independent (<25% sequence identity) to the two datasets which were used to build I-Mutant2.0 [8] and MUpro [9]. Therefore, we used S236 for independent testing and comparison with related work. Another independent test set, S543 (543 mutations in 55 proteins), was compiled as a subset of the 2648 mutations from Dehouck *et al.* [3]. S543 has < 25% sequence identity to both S1676 and S236. Supplementary Figure S5 shows the distributions of the experimentally measured $\Delta\Delta G_u$ for the three datasets. Supplementary Figures S6, S7, and S8 show the frequencies and EASE-MM's prediction errors of all wild-type and mutant amino acid types for S1676, S543, and S236, respectively.

To see if the comparison with structure-based energy functions (Rosetta [5], FoldX [4], DFIRE [7], and PoPMuSiC [3]) was affected by structures determined with nuclear magnetic resonance (NMR), we also compiled the S157 (a subset of S236 comprising 157 mutations in 16 proteins) and S405 (a subset of S543 comprising 405 mutations in 44 proteins) datasets of high-resolution (≤3 Å) crystal structures from the Protein Data Bank [20].

Finally, to study the relationship between the predicted $\Delta\Delta G_u$ and human germline non-synonymous SNVs, we compiled a dataset of 10,511 disease-causing (2201 proteins) and 278,760 putatively neutral (20,096 proteins) SNVs from ClinVar [21] and 1000 Genomes Project [22], respectively. For the distribution analysis, we considered the subset comprising 50,910 putatively neutral SNVs (14,113 proteins) with AF ≥ 1%.

Predictive features

We employed three types of predictive features in our method: evolutionary conservation, amino acid parameters, and predicted structural properties. To estimate evolutionary conservation of the mutation site, we used three iterations of PSI-BLAST [36] on the NCBI non-redundant database with an *e*-value threshold of 10^{-3}. From the PSSM generated with PSI-BLAST, we extracted the probability of the wild-type ($PSSM_{wt}$) and mutant ($PSSM_{mt}$) amino acids at the mutation site. We implemented two different features, $PSSM_{wt}$ and $\Delta PSSM = PSSM_{mt} - PSSM_{wt}$. We also included a feature encoding the overall conservation of the mutation site as property entropy (PE) with respect to six sets grouping amino acids based on their chemical properties as aliphatic (A, V, L, I, M, C), aromatic (F, W, Y, H), polar (S, T, N, Q), positive (K, R), negative (D, E), and special (G, P) [37]. The property entropy was calculated from a multiple sequence alignment of the 30 most similar sequences from the NCBI non-redundant database ranked by *e*-value with PSI-BLAST (using 100, 500, or all sequences resulted in a lower correlation with $\Delta\Delta G_u$, S1676 dataset). We used the implementation from Capra and Singh [38] to calculate the property entropy based on the following equation:

$$\text{PE}(msa_i) = 1 - \left(-\frac{\sum_{g \in G} p(msa_i, g) \times \log p(msa_i, g)}{\log |msa_i|} \right),$$

$$p(msa_i, g) = \sum_{aa \in g} p(msa_i, aa),$$

where msa_i is the *i*-th column of the multiple sequence alignment *msa*, *G* is the set of the defined property groups, and $p(msa_i, g)$ is the probability of the property group *g* at msa_i, which is equal to the sum of probabilities of the amino acid types (*aa*) belonging to *g*.

Different amino acid parameters have been used for the prediction of stability changes [39,40,41,42]. We adopted a total of 11 amino acid parameters: hydrophobicity, volume, polarisability, isoelectric point, helix tendency, sheet tendency, and a steric parameter (graph shape index) from Meiler *et al.* [43]; compressibility, bulkiness, and equilibrium constant with reference to the ionisation property of COOH group from Gromiha *et al.* [44]; and flexibility from Vihinen *et al.* [45]. For each amino acid parameter (AAP), we calculated $\Delta AAP = AAP_{mt} - AAP_{wt}$, where AAP_{mt} and AAP_{wt} denote the value of the given AAP for the mutant and wild-type amino acids, respectively. Supplementary Table S5 provides the values of the 11 parameters for the 20 common amino acids.

Finally, we considered five structural features predicted from the protein sequence: rASA, helix, sheet, coil, and disorder probabilities. The rASA and SS probabilities were predicted using SPIDER [16]. The disorder probability was calculated using SPINE-D [46].

Feature selection and multiple models

To build the five models employed by EASE-MM, we partitioned the S1676 training dataset according to SS (helix, sheet, and coil) and ASA (buried or exposed with a 25% threshold). SS and ASA were predicted from the protein sequence with SPIDER [16]. Supplementary Figure S5 shows the distributions of the experimentally measured $\Delta\Delta G_u$ for the different data partitions.

A unique set of features was identified for each of the five SVM models using the SFFS algorithm [17]. SFFS starts with an empty set of features S_0 and iteratively searches for a better set of features in two steps. First, the best feature f is selected as the one for which $S_i = S_{i-1} \cup \{f\}$ yields the lowest RMSE. Second, features f^* for which $S_i - \{f^*\}$ yields a lower RMSE than S_{i-1} are iteratively removed. Thus, the number of features in S is not monotonously increasing because the search is floating up and down. Supplementary Table S2 lists the selected features for each EASE-MM model.

Training and evaluation

We employed the S1676 dataset to design our method, perform feature selection, and optimise all parameters using the *unseen-protein* 10-fold cross-validation. The *unseen-protein* cross-validation is used to avoid over-fitting on specific proteins by splitting the dataset into cross-validation folds so that all mutations of a cluster of similar proteins (≥25% sequence identity) are always contained within a single fold. These clusters were identified with Blastclust [47]. To devise a robust estimate of the prediction performance, we replicated the cross-validation procedure 100 times with randomly re-generated folds and averaged the results.

We implemented EASE-MM with ε-SVR (support vector regression) and radial basis function (RBF) kernel using the LibSVM [48] library. For ε-SVR, we optimised three parameters (C, γ, and ε) using a grid search in the range of $C \in \{2^{-1}, 2^0, \ldots, 2^6\}$, $\gamma \in \{2^{-8}, 2^{-7}, \ldots, 2^0\}$, and $\varepsilon \in \{2^{-8}, 2^{-7}, \ldots, 2^{-1}\}$. Then, each model was trained on S1676 and tested independently using S543 and S236 to confirm that our approach did not result in over-fitting. Importantly, S543 and S236 did not share similar sequences (≥25% sequence identity) with S1676 and were not used during the design, feature selection, or parameter optimisation in any way. Furthermore, S543 and S236 were disjoint with < 25% sequence identity. The performance of EASE-MM was assessed in terms of Pearson correlation coefficient (r) and root mean square error (RMSE):

$$r = \frac{n\sum x_i y_i - \sum x_i \sum y_i}{\sqrt{n\sum x_i^2 - (\sum x_i)^2}\sqrt{n\sum y_i^2 - (\sum y_i)^2}},$$

$$\mathrm{RMSE} = \sqrt{\frac{1}{n}\sum (x_i - y_i)^2}.$$

Finally, we re-optimised the ε-SVR parameters and trained the final EASE-MM models, which are deployed on our web-server, using a joint S1676 + S236 dataset in order to maximise the size of the training data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2016.01.012.

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Abbreviations used:
AF, allele frequency; ASA, accessible surface area; SFFS, sequential forward floating selection; SNV, single nucleotide variant; SS, secondary structure; SVM, support vector machine; SVR, support vector regression.

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