Predicting protein thermal stability changes
upon point mutations using statistical potentials:
Introducing HoTMuSiC

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Abstract

The accurate prediction of the impact of an amino acid substitution on the thermal stability of a protein is a central issue in protein science, and is of key relevance for the rational optimization of various bioprocesses that use enzymes in unusual conditions. Here we present one of the first computational tools to predict the change in melting temperature $\Delta T_m$ upon point mutations, given the protein structure and, when available, the melting temperature $T_m$ of the wild-type protein. The key ingredients of our model structure are standard and temperature-dependent statistical potentials, which are combined with the help of an artificial neural network. The model structure was chosen on the basis of a detailed thermodynamic analysis of the system. The parameters of the model were identified on a set of more than 1,600 mutations with experimentally measured $\Delta T_m$.

The performance of our method was tested using a strict 5-fold cross-validation procedure, and was found to be significantly superior to that of competing methods. We obtained a root mean square deviation between predicted and experimental $\Delta T_m$ values of 4.2°C that reduces to 2.9°C when ten percent outliers are removed. A webserver-based tool is freely available for non-commercial use at soft.dezyme.com.

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Introduction

The possibility of rationally modifying protein sequences to increase their thermal resistance is a main goal in protein engineering. Indeed, the design of new enzymes and other proteins that remain stable and active at temperatures that differ from their physiological temperatures would allow the optimization of a wide series of biotechnological processes in many sectors such as agro-food, biopharmaceuticals and environment.

Unfortunately, it is extremely complicated to predict the effect of mutations on the thermal stability of proteins, defined through the melting temperature $T_m$, i.e. the temperature at which the protein undergoes the reversible (un)folding transition. It is even more difficult than predicting the change in thermodynamic stability defined by the folding free energy $\Delta G(T_r)$ at room temperature ($T_r$), since it requires a precise understanding of the variation of the free energy $\Delta G(T)$ as a function of the temperature ($T$) of the different types of interactions that contribute to protein stability, i.e. between the various chemical groups that form the solvent and the 20 amino acids. This is a longstanding problem that is currently far from being solved. The analyses performed in the last thirty years led to the conclusion that there is no unique and specific factor that ensures an enhancement of the thermal stability of all proteins, but that there is - though very approximately - such a factor inside each protein family, as homologous proteins tend to involve the same kinds of stabilizing interactions (see for example [1–10] and references therein).

A series of experimental approaches have been developed to design new mutants with higher or lower melting temperature than the wild-type protein. They are mostly based on directed evolution experiments that mimic natural evolution, sometimes in combination with computational approaches (see [11, 12] and references therein). Unfortunately, these methods are only partially successful. Indeed, they are expensive and time-consuming, and moreover limited by the vastness of sequence space and the low frequency of the thermally stabilizing mutations.

In silico protein engineering constitutes an alternative for the design of new proteins with modified stability. Different computational tools based on a variety of approaches and information including residue conservation, energy estimations as well as structural, sequence and dynamical features, have been developed to get a prediction of the thermodynamic stability changes upon point mutations defined through $\Delta \Delta G(T_r)$, the difference of folding
free energy $\Delta G$ between the mutant and wild-type proteins at room temperature. Quite interestingly, some of these methods can reach a good accuracy at very low computational cost, with the sole knowledge of the wild-type structure [13–23] (see also [24] for a comparison of their performances). This makes the fastest among them ideal tools for stability analyses of the entire structurome. One of the major drawbacks of these methods is that the results are usually biased towards the training datasets even if strict cross validation is applied, which makes the estimation of their true performances an almost impossible task [25].

The impact of point mutations on the thermal stability, defined through $\Delta T_m$ which measures how the protein melting temperature changes upon mutations, has been much less investigated than the thermodynamic stability, as it is more intricate and requires taking into account that the amino acid interactions are temperature dependent. Therefore, there are very few in silico tools for predicting $\Delta T_m$ [16, 26–28]. The common strategy to study the enhancement of thermal resistance is to assume the thermodynamics and thermal stabilities to be perfectly correlated (or $\Delta \Delta G(T_r)$ and $\Delta T_m$ to be perfectly anticorrelated). Unfortunately, even if this approximation can be used in a first instance, it is not always reliable [29]. As a consequence, the predictions of $\Delta T_m$ are in general less accurate because the intrinsic errors on the $\Delta \Delta G$ predictions have to be summed with the errors due to the imperfect correlation of the two stabilities. As an example, the anticorrelation between $\Delta \Delta G$’s predicted using the thermodynamic stability change predictor PoPMuSiC [22] and measured $\Delta T_m$’s is not so satisfactory and is equal to 0.51, whereas the correlation between predicted and experimental $\Delta \Delta G$’s is 0.63.

For all these reasons, it is necessary to design a specific computational tool for predicting $\Delta T_m$ in a fast and more precise way. This is the aim of the present paper, in which we present a new, knowledge- and thermodynamics-based, method called HoTMuSiC, which is able to predict this quantity using as sole input data, the three-dimensional (3D) structure of the wild-type protein and – when available – its melting temperature $T_m$. A very preliminary version of this method has been published in [30]. The main reasons of the success of HoTMuSiC are rooted on the one hand in the thorough physical analysis of the system which helped correct guessing the form of the model structures, and on the other hand in the use of temperature-dependent statistical potentials [22, 31–33] that are extracted from non-redundant datasets of protein X-ray structures of thermostable and mesostable proteins. We would like to emphasize that these are presently the only potentials that yield
an estimation of the temperature dependence of the folding free energy contributions of the different amino acid interactions – albeit in an approximate, effective, manner.

Results

Theoretical analysis

The thermodynamic stability change upon mutation is measured by $\Delta \Delta G(T_r)$, i.e. the difference between the Gibbs folding free energies of the mutant ($\Delta G^{\text{mut}}$) and wild-type ($\Delta G^{\text{wild}}$) proteins at the reference temperature $T_r$:

$$\Delta \Delta G(T_r) = \Delta G^{\text{mut}}(T_r) - \Delta G^{\text{wild}}(T_r).$$

(1)

Usually $T_r$ is taken as the room temperature: $T_r = 298 K$. $\Delta \Delta G$'s upon point mutations can be predicted in silico using a series of tools [13–23], which reach a relatively good accuracy with a standard deviation between the experimental and predicted $\Delta \Delta G$'s of 1 to 2 kcal/mol.

Less prediction methods have been developed for the change in thermal stability upon mutations, measured by $\Delta T_m$, i.e. the difference between the melting temperature of the mutant ($T_m^{\text{mut}}$) and wild-type ($T_m^{\text{wild}}$) proteins:

$$\Delta T_m = T_m^{\text{mut}} - T_m^{\text{wild}}.$$ 

(2)

In a first approximation the two protein stabilities can be assumed to be strongly interdependent. Indeed, focusing on two-state folding transitions and assuming: (1) the mutations to be small perturbations with respect to the wild-type state; (2) the folding heat capacity $\Delta C_P$ to be $T$-independent; (3) its variations upon mutations to vanish ($\Delta \Delta C_P \equiv \Delta C_P^{\text{mut}} - \Delta C_P^{\text{wild}} = 0$) and (4) similarly for the folding enthalpy ($\Delta \Delta H_m \equiv \Delta H^{\text{mut}}(T_m^{\text{wild}}) - \Delta H^{\text{wild}}(T_m^{\text{wild}}) = 0$), one can derive the simple relation:

$$\Delta \Delta G(T_r) \simeq \frac{\Delta H_m T_r}{(T_m^{\text{wild}})^2} \Delta T_m,$$

(3)

with $\Delta H_m \equiv \Delta H^{\text{wild}}(T_m^{\text{wild}}) = \Delta H^{\text{wild}}(T_r) + \Delta C_P(T_m^{\text{wild}} - T_r)$. This equation directly relates the two stabilities, however with a coefficient that depends on the thermal characteristics of the wild-type protein through its melting temperature $T_m^{\text{wild}}$ and its folding free enthalpy.
Note that, since the enthalpy change upon folding is negative, $\Delta \Delta H$ and $\Delta T_m$ are anticorrelated, as expected.

Unfortunately, the situation is in general less simple, especially for highly destabilizing or highly stabilizing mutations, and we have to take into account that the enthalpy and heat capacity variations do not vanish, i.e. $\Delta \Delta H_m \neq 0 \neq \Delta \Delta C_P$. This is illustrated by the fact that the correlation coefficient between experimental $\Delta T_m$’s and $\Delta \Delta G$’s is about $-0.8$, which signals an imperfect correlation between these quantities. In this case, and still assuming $\Delta C_P$ to be $T$-independent, Eq. (3) becomes:

$$\Delta \Delta G(T_r) \simeq \frac{\Delta H_m}{(T_m^{\text{wild}})^2} \Delta T_m + \Delta \Delta H_m \left[ 1 - \frac{T_r}{T_m^{\text{wild}}} + \frac{T_r}{(T_m^{\text{wild}})^2} \Delta T_m \right] + \Delta \Delta C_P \left[ T_r - T_m^{\text{wild}} - T_r \log \frac{T_m}{T_m^{\text{wild}}} \right] + O \left( \frac{(\Delta T_m)}{(T_m^{\text{wild}})^2} \right). \tag{4}$$

Thus the simple relation between the two descriptors of protein stability is lost: the proportionality coefficient can be positive or negative, and becomes also mutation-dependent in addition of being protein-dependent.

It is easier to understand the meaning of the possible types of correlations between the two stabilities with a graphical representation. If the assumption of small perturbation and as a consequence Eq. (3) holds, the full protein stability curve changes upon mutation like in Fig. 1.a. Typically, in this case, the wild-type has one interaction more than the mutant (or conversely). Otherwise the scenario is more similar to Fig. 1.b, where one cannot say a priori which type of connection there is between $\Delta \Delta G(T_r)$ and $\Delta T_m$ without the knowledge of additional information. It can for example occur when an interaction that is more stabilizing at room temperature is replaced by another that is more $T$-resistant thereby modifying the $\Delta H_m$, or when a change in the 3D structure occurs which modifies the protein’s solvent accessible surface area and thus the $\Delta C_P$.

In a first approach to the prediction of $\Delta T_m$ upon mutations, we consider the small perturbation approximation and thus Eq. (3) as valid and compute $\Delta T_m$ as the sum of usual, temperature-independent, statistical potential contributions. In a second approach, which is expected to be more accurate for highly destabilizing or stabilizing mutations, we use $\Delta \Delta G$-values calculated at different temperatures. Note however that structural modifications are more likely to occur in such case, and thus that the accuracy of the energy evaluations on
FIG. 1: Effect of mutations on the full stability curve of an hypothetical protein. (a) For a small perturbation of the wild-type state, we observe a negative proportionality between $\Delta T_m$ and $\Delta \Delta G$; (b) Such simple relation is lost for highly destabilizing or stabilizing mutations.

the basis of the wild-type structure alone is questionable. For the purpose of estimating $\Delta \Delta G(T)$, we use the formalism of the temperature-dependent statistical potentials introduced in [31–33].

Construction of the model

*Standard and temperature-dependent statistical potentials*

The standard formalism of statistical potentials [34–36] has been fruitfully applied to a variety of analyses that range from the prediction of protein structure, stability, and protein-protein and protein-ligand binding affinities. It basically consists in deriving a potential of mean force (PMF) from frequencies of associations of structure and sequence elements in a dataset of protein X-ray structures. Under some approximations whose validity has been discussed [36–40] and making use of the Boltzmann law, the simplest PMF can be written as:

$$\Delta W(s,c) \equiv -kT \ln \frac{F(s,c)}{F(s)F(c)} = -kT \ln \frac{n(c,s)n}{n(c)n(s)}, \quad (5)$$

where $c$ and $s$ are structure and sequence elements respectively, $F$ represent the relative frequencies of $c$ and/or $s$ which are expressed as a function of the number of occurrences $n$, $k$ is the Boltzmann constant and $T$ the absolute temperature. Following [41], higher order
| Type               | Standard potentials       | \(T\)-dependent potentials                  |
|--------------------|---------------------------|---------------------------------------------|
| Distance           | \(\Delta W_{sd}\); \(\Delta W_{sdS}\) | \(\Delta W_{sd}^{MT}\); \(\Delta W_{sdS}^{MT}\) |
| Distance/Accessibility | \(\Delta W_{sad}\)           | \(\Delta W_{sad}^{MT}\)               |
| Distance/Torsion   | \(\Delta W_{std}\)            | \(\Delta W_{std}^{MT}\)               |
| Accessibility      | \(\Delta W_{sa}\); \(\Delta W_{saa}\) | \(\Delta W_{sa}^{MT}\) + \(\frac{1}{2}(\Delta W_{saa}^{MT} + \Delta W_{saa}^{MT})\) |
| Torsion            | \(\Delta W_{st}\); \(\Delta W_{sst}\); \(\Delta W_{stt}\) | \(\Delta W_{st}^{MT}\) + \(\frac{1}{2}(\Delta W_{stt}^{MT} + \Delta W_{sst}^{MT})\) |

TABLE I: List of 9 \(T\)-independent and 5 \(T\)-dependent statistical potentials used in the \(\Delta T_m\)-prediction methods. The subscripts \(M/T\) indicate that the potentials are extracted from either the mesostable (\(M\)) or thermostable (\(T\)) protein subset.

potentials can be constructed by considering more than two structure elements and/or sequence elements. Considering for example two sequence elements \(s\) and \(s'\) and one structure element \(c\), the above expression of PMF gets modified as:

\[
\Delta W(s, s', c) \approx -kT \ln \frac{F(s, s', c)}{F(s, s') F(s', c) F(s, c)} = -kT \ln \frac{n(s, s', c) n^2}{n(s, s') n(s, c) n(s', c)}. \tag{6}
\]

Using Eqs (5-6) and their generalizations defined in [41], we derived 9 different statistical potentials from a dataset of about 4,100 proteins with well-resolved 3D structure and low sequence similarity; they are listed in Table I. They differ by the number of sequence and/or structure elements involved and by their type. Each sequence element \(s\) is an amino acid type at a given position and each structure element \(c\) is either the spatial distance \(d\) between two residues, the backbone torsion angle domain \(t\) or the solvent accessibility \(a\) of a residue.

The statistical potential formalism has recently been extended to include more properly the thermal characteristics of proteins and in particular the fact that amino acid interactions are temperature-dependent [9, 31–33]. Following this approach, a dataset of about 170 proteins with known melting temperature and 3D structure was used and divided into two subsets, one with only mesostable proteins (with \(T_m\) less than about 65°C), and the other with thermostable proteins (with \(T_m\) higher than about 65°C). A series of 9 different potentials were extracted from each subset, which are listed in Table I. To limit the number of parameters to be optimized (see next section) and thus to avoid overfitting as much as possible, these twelve potentials were combined into five potentials (Table I).
As expected, these $T$-dependent potentials reflect the thermal characteristics – mesostable or thermostable – of the subset from which they are derived: the former set better describes the interactions in the low temperature region while the second set better reflects the thermal properties at high temperatures. This approach has shown good performances in the prediction of the thermal resistance and of the stability curve as a function of the temperature of proteins that belong to the same homologous family [32, 33].

The structure elements defining these potentials are the same as for the temperature-independent potentials. In contrast, the size of the mesostable and thermostable protein datasets is much smaller than the dataset used for the standard statistical potentials, as it requires the knowledge of the melting temperature. To deal with the smallness of the datasets, we used several tricks that consists of corrections for sparse data and the smoothing of the potentials, following [9, 31–33] .

In addition to the standard and $T$-dependent potentials $\Delta W$, we also considered volume terms in the folding free energy estimation, which are defined as the volume difference $\Delta V$ between the mutant and wild-type amino acids [22, 23]. In order to take into account that the creation of a cavity in the protein interior ($\Delta V < 0$) can have a different impact on the stability compared to the addition of stress ($\Delta V > 0$), we introduced two separate terms ($\Delta V_-$) and ($\Delta V_+$) defined as $\Delta V_\pm = \theta(\pm \Delta V)\|\Delta V\|$ where $\theta$ is the Heaviside function.

Artificial neural networks and parameter identification

The above-defined potential terms were combined to predict how the melting temperature changes upon mutations, using two different model structures. The second model (called $T_m$-HoTMuSiC) uses the $T_m$ of the wild-type, while the first (HoTMuSiC) does not. To identify the parameters involved in these combinations, we used an artificial neural network (ANN) with peculiar activation functions.

In the first approach (HoTMuSiC), we assumed that $\Delta T_m$ can be written as the sum of twelve contributions, the nine energy terms $\Delta \Delta W_\nu = \Delta W_{\nu}^{\text{wild}} - \Delta W_{\nu}^{\text{mut}}$ ($\nu = 1, \ldots, 9$) computed from the standard, $T$-independent, statistical potentials listed in Table 1, the two volume terms and an "independent" term that only depends on the solvent accessibility. The functional form reads as:
\[ \Delta T_{m}^{\text{HoT}} = \frac{1}{aN_r + c} \left( \sum_{\nu=1}^{9} \alpha_{\nu}(A) \Delta W_{\nu} + \alpha_+ (A) \Delta V_+ + \alpha_- (A) \Delta V_- + \alpha_I (A) \right), \]  
where \( N_r \) is the number of residues in the protein, and the coefficients \( \alpha_{\nu}(A) \) were chosen to be sigmoid functions of the solvent accessibility \( A \) of the mutated residues:

\[
\alpha_{\nu}(A) = \frac{\omega_{\nu}}{1 + e^{r_{\nu}(A-b_{\nu})}} + \phi_{\nu},
\]

with \( a, c, \omega_{\nu}, r_{\nu}, b_{\nu}, \phi_{\nu} \in \mathbb{R} \). We have chosen the activation functions to be sigmoidal since they model a smooth transition from the protein core to the surface, and since the weight of the different energy contributions have been shown to differ in these two regions [22][47].

To identify the fifty parameters introduced in Eq. (7), we have chosen a standard feed-forward ANN with one input and one output layer (schematically depicted in Fig. 2a). The cost function to be minimized is the mean square deviation between the experimental and predicted values of \( \Delta T_{m} \) for the dataset \( S_{\text{mut}} \) that contains \( N_{\text{mut}}=1,626 \) mutations for which an experimental \( \Delta T_{m} \)-value is available (see Methods section):

\[
\sigma^2 = \frac{1}{N} \sum_{\ell=1}^{N} (\Delta T_{m\ell}^{\exp} - \Delta T_{m\ell}^{\text{pred}})^2.
\]

The initial values of the weights were chosen randomly. To take into account that the ANN training algorithm can get stuck in local minima, the initialization and training processes were repeated about thirty times and the solution reaching the lowest \( \sigma \)-value was chosen [43, 44].

We used a strict five-fold cross validation procedure with an early stopping procedure for evaluating the method’s performance. More precisely, the entire set of mutations was split randomly into a training set (containing 90% of the mutations) and a test set (with the remaining 10%). The training set was then further randomly split into a smaller set (with 80% of the mutations) on which the parameters were identified, and a validation set (with 10% mutations) on which the early stop threshold was determined, namely the maximum number of iterations in the gradient descent procedure before its convergence [45, 46]. This early stop is necessary to avoid overfitting, as in general the network starts to learn too much from the training dataset after a certain number of iterations, with the consequence
that the error in cross validation starts to raise. As a final step in the computation, the prediction error $\sigma$ is independently calculated on the test set.

FIG. 2: Schematic representation of the ANN’s used for the parameter identifications. (a) HoT-MuSiC method: 2-layer ANN, corresponding to a perceptron with sigmoid activation functions and 12 input neurons encoding the 9 $T$-independent potentials specified in Table I, two volume terms and an independent term; (b) $T_m$-HoTMuSiC method: 3-layer ANN, consisting of 3 perceptrons with sigmoid weights; the first perceptron has 5 input neurons encoding the 5 mesostable potentials listed in Table I, the second has 5 input neurons corresponding to the 5 thermostable potentials, and the last perceptron has 3 neurons for the volume and independent terms. The outputs of these three perceptrons (Meso, Thermo, and Vol+I) are the inputs of another perceptron with polynomial weight functions.

In the second method for predicting $\Delta T_m$, called $T_m$-HoTMuSiC, we used quite a different approach, with as building blocks the $T$-dependent statistical potentials listed in Table I and the $T_m$ wild of the wild-type protein. More precisely, Eq. (7) describing the $\Delta T_m$ functional was modified into:

$$\Delta T_m^{\text{pred}} = \beta^T(T_m^{\text{wild}}) \sum_{\nu=1}^5 \alpha_\nu(A) \Delta \Delta W^T_\nu + \beta^M(T_m^{\text{wild}}) \sum_{\nu=1}^5 \alpha_\nu(A) \Delta \Delta W^M_\nu + \beta^V(T_m^{\text{wild}}) [\alpha_+(A) \Delta V_+ + \alpha_-(A) \Delta V_- + \alpha_l(A)]$$

(10)

where the $\alpha_\nu(A)$ parameters are sigmoid functions of the solvent accessibility $A$ (Eq. (8)) and the three functions $\beta^T(T_m^{\text{wild}})$, $\beta^M(T_m^{\text{wild}})$ and $\beta^V(T_m^{\text{wild}})$ are polynomial functions of the
melting temperature of the wild-type protein and its number of residues \(N_r\). Their functional form is guessed from Eq. (3) and approximated as:

\[
\beta'(T_{\text{wild}}^m) = \gamma' (T_{\text{wild}}^m)^2 + \delta' T_{\text{wild}}^m + \xi',
\]

with \(I = T\) (thermostable), \(M\) (mesostable) or \(V\) (volume). The dependence on the number of residues \(N_r\) comes from the enthalpy factor \(\Delta H_m\) in Eq. (3), as these two quantities show a good correlation in a first approximation [48].

To identify the 67 parameters of this second method, an ANN with an input layer, a hidden layer and an output layer is used, as shown schematically in Fig. 2b. The input layer consists of three sets of neurons, one set encoding the mesostable potentials, a second one the thermostable potentials, and a third one the volume and independent terms. Three perceptrons use these three sets of input neurons and generate the three output signals of the hidden layer. These are the input of yet another perceptron, which yields the \(\Delta T_m^r\)-prediction as output. The initialization and identification procedures of all the weights and the cross validation procedure are the same as for the first method.

The final \(\Delta T_m\) predictions of the \(T_m\)-HoTMuSiC method are defined as the mean of the two predictions given by Eqs (10) and (7):

\[
\Delta T^{T_m\text{-HoTMuSiC}} = \frac{1}{2} \left( \Delta T^{\text{HoTMuSiC}} + \Delta T^{\text{pred}} \right).
\]

**Performance of HoTMuSiC and \(T_m\)-HoTMuSiC**

The values of the root mean square deviation \(\sigma\) between measured and predicted \(\Delta T_m\) values (Eq. 9), computed in strict cross validation, are shown in Table II. For HoTMuSiC, we obtained \(\sigma = 4.3^\circ\text{C}\); the Pearson correlation coefficient \(r\) between experimental and predicted \(\Delta T_m\)’s is 0.59. The performance of the \(T_m\)-HoTMuSiC version is slightly better with \(\sigma = 4.2^\circ\text{C}\) and \(r = 0.61\). When ten percent outliers are excluded, \(\sigma\) decreases to 2.9\(^\circ\text{C}\) and \(r\) rises to 0.75. The results are plotted in Fig. 3.

The \(T_m\)-HoTMuSiC version that encodes information about the melting temperature of the wild-type protein and uses \(T\)-dependent potentials thus performs slightly better than the \(T\)-independent version, but not as much as could be expected on the basis of earlier analyses [32][33]. Indeed, in principle, the mesostable potentials should describe the mutations in
FIG. 3: Experimental $\Delta T_m$ values versus predicted $\Delta T_m^{HoT}$ (left, $r=0.59$) and $\Delta T_m^{TmHoT}$ (right, $r=0.61$) values. The straight lines are the bisectors of the first and third quadrants. The temperatures are in Celsius degrees ($^\circ$C).

the mesostable proteins much better than the thermostable potentials and vice-versa. The reasons for this mitigated result could be due to the lower accuracy of the $T$-dependent potentials compared to the standard ones since they are extracted from smaller protein sets. Or they could be rooted in the information loss upon the reduction of the number of potentials (see Eq. (7) versus Eq. (10)), which is done to avoid overfitting issues.

Moreover, we can see from Table III and Fig. 4 that the $\sigma$-value for amino acid substitutions in the core ($A<15\%$) and partially buried positions ($15\%<A<50\%$) is on the average larger than that of surface mutations ($A>50\%$). In contrast, the correlation coefficient $r$ is higher in the core and in the partially buried region and smaller at the surface. This apparent discrepancy is in fact due to the higher variance of $\Delta T_m$ in the core, which drives the correlation and increases the value of $r$. In the surface region, the predictions are more accurate (lower $\sigma$) but the $\Delta T_m$ variance and the correlation coefficient are lower. When normalizing $\sigma$ by the standard deviation of $\Delta T_m$, we obtain values that increase from the core to the surface (see Table III).

**Comparison with other $\Delta T_m$ predictors**

As far as we known, only two other $\Delta T_m$ predictors are described in the literature, which are strongly different from ours. The strategy of Saraboji et al. [28] consists in predicting for a mutation from wild-type residue W to mutant M the mean value of the $\Delta T_m$’s of all
FIG. 4: Experimental $\Delta T_m$’s versus predicted $\Delta T_m^{HoT}$ values for mutations in different protein regions: core ($A < 15\%$), partially buried ($15\% < A < 50\%$), and surface ($A > 50\%$). The straight lines are the bisectors of the first and third quadrants. The temperatures are in °C.

| Prediction Tool | $\sigma$ (°C) | $r$ | $\sigma^*$ (°C) | $r^*$ | $N_{mut}$ |
|-----------------|---------------|-----|-----------------|------|----------|
| HoTMuSiC        | 4.3           | 0.59| 2.9             | 0.75 | 1626     |
| $T_m$-HoTMuSiC  | 4.2           | 0.61| 2.9             | 0.75 | 1626     |

TABLE II: Scores of HoTMuSiC and $T_m$-HoTMuSiC; $\sigma^*$ and $r^*$ correspond to $\sigma$ and $r$ with 10% outliers removed.

The analogous mutations $W \rightarrow M$ in the training dataset. A similar strategy proposed in the same work is based on the classification of the mutations in terms of the secondary structure and solvent accessibility of the wild-type residues and predicts as $\Delta T_m$ the mean of the experimental $\Delta T_m$’s occurring in the suitable class in the learning set. A limitation of this approach is that not all wild-type to mutant mutations are present in the learning set due to the lack of experimental data. The second method is called AutoMute [16, 26, 27] and is based on residue environment scores. It proceeds by reducing protein 3D structures to ensembles of $C_\alpha$ atoms, and applying Delaunay tessellation to identify quadruplets of nearest neighbor residues. A log-likelihood potential is constructed from the number of occurrences of the quadruplets in a dataset of 3D structures and then used as the key ingredient in the computation of $\Delta T_m$. 

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Solvent accessibility $\sigma$ ($^\circ$C) $r$ $\frac{\sigma}{\sqrt{\langle (\Delta T_m)^2 \rangle}}$ $N_{\text{mut}}$

| Solvent accessibility | $\sigma$ ($^\circ$C) | $r$ | $\frac{\sigma}{\sqrt{\langle (\Delta T_m)^2 \rangle}}$ | $N_{\text{mut}}$ |
|-----------------------|----------------------|-----|---------------------------------|-----------------|
| A<15%                 | 4.9                  | 0.70 | 0.66                           | 734             |
| 15%<A<50%             | 4.2                  | 0.57 | 0.81                           | 513             |
| A>50%                 | 2.8                  | 0.54 | 0.83                           | 379             |

TABLE III: Scores of $T_m$-HoTMuSiC as a function of the solvent accessibility A of the mutated residues.

| Prediction Tool     | $\sigma$ ($^\circ$C) | $r$ | $N_{\text{mut}}$ |
|---------------------|----------------------|-----|-----------------|
| $T_m$-HoTMuSiC      | 3.7                  | 0.65 | 630             |
| AutoMute v2.0 [16]  | 4.7                  | 0.42 | 607             |
| Saraboji [28]       | 5.4                  | 0.25 | 580             |

TABLE IV: Comparison between the performances of HotMuSiC and those of the two other methods evaluated in cross-validation on the dataset $S_{\text{sub}}$.

To make a cross-validated comparison between our results and those of these two methods, we have chosen a subset $S_{\text{sub}}$ of our dataset $S_{\text{mut}}$ consisting of the mutations that are not present in the AutoMute learning set (see [60] for a list), and trained versions of $T_m$-HoTMuSiC and the Saraboji method on the set $S_{\text{mut}} \setminus S_{\text{sub}}$. The performances of the three methods are reported in Table IV. $T_m$-HoTMuSiC shows the best performance, with an improvement of about 20% and 30% with respect to AutoMute [16] and the Saraboji method [28], respectively. The $\sigma$-values computed on the $S_{\text{sub}}$ set are equal to 3.7, 4.7 and 5.4 $^\circ$C for $T_m$-HoTMuSiC, AutoMute and Saraboji method, respectively. Note that the performance of the latter two methods has been evaluated on a slightly reduced dataset, since the $\Delta T_m$ values could not be computed for some of the mutations.

Discussion

We developed a thermodynamics-based and knowledge-driven $\Delta T_m$ prediction method that does not exploit the common assumption of a perfect correlation between thermal and thermodynamics stabilities. The basic ingredients of our approach include standard
and $T$-dependent statistical potentials that are combined through the use of ANN’s. The performance in cross validation of the two versions of our method, HoTMuSiC and $T_m$-HotMuSiC (which requires the $T_m$ of the wild type), are quite good with a $\sigma$-value of 4.3 and 4.2 °C, respectively, which goes down to 2.9 °C upon removal of 10% outliers. They perform significantly better, by 20 to 30%, than the two other $\Delta T_m$ prediction methods described in the literature.

HoTMuSiC and $T_m$-HotMuSiC are accessible via the webserver soft.dezyme.com and are free for non-commercial use. They are extremely fast and allow the $\Delta T_m$ predictions for all possible single-site mutations in a protein in a few minutes. This webserver is presented in an application note [49].

Our software thus yields quite accurate results, and allows rapid screening of all possible point mutations in a protein structure and identifying a subset that is likely to yield the required thermal resistance. This subset must then be analyzed further, either by using more detailed computational techniques, or by experimental means. HoTMuSiC and $T_m$-HotMuSiC are very useful and user-friendly tools for every researcher who wishes to rationally design modified proteins with controlled characteristics.

Notwithstanding the large applicability and good accuracy of HoTMuSiC and $T_m$-HotMuSiC, it is worth discussing their limitations and the sources of errors that affect the predictions. These are:

- The wild-type and mutant structures are supposed to be identical (up to the substituted side chain) and the possible structural modifications are only encoded in the volume terms $\Delta V_\pm$; local structure rearrangements upon residue substitutions, for example in the hydrophobic core, yet depend on many more parameters such as the residue depth and the backbone flexibility [50–54].

- The mutation dataset is strongly unbalanced towards destabilizing mutations, which is likely to add unwanted hidden biases even if a strict cross validation procedure is applied [25]. Only when more stabilizing mutations will be experimentally characterized will we be able to completely exclude the biasing impact of this stabilizing-destabilizing asymmetry.

- The experimental conditions at which the $T_m$ measurements are performed usually differ in terms of pH, ion concentration or buffer composition, which induces noise
in the learning set and errors in the predictions. To limit this problem, we chose the entries derived from experiments performed at pH as close as possible to seven, and made a weighted average of the experimental $\Delta T_m$'s of a same mutation, when available.

- The $T$-dependent potentials suffer from the smallness of the dataset of protein structures with known $T_m$. Different tricks have been used to limit this issue.

- The possible parameter overfitting is like always an important concern, especially for the $T_m$-HoTMuSiC version, in which the number of potentials is three times larger than for HoTMuSiC. To avoid overfitting, we decided to decrease the number of parameters in $T_m$-HoTMuSiC by fixing some coefficients of the linear combination of potentials (see Table I).

Different ways will be explored in an attempt to further improve the prediction performances of HoTMuSiC. They obviously include the enlargement of the datasets of proteins of known structure and $T_m$, and of the mutations of known $\Delta T_m$. We will also investigate different ANN architectures, the addition of hidden layers, and the inclusion of other features such as the change in conformational flexibility upon mutation, which seems related to the thermal stability even if a quantitative connection between the two quantities on a large scale is still missing [55–58]. Finally, it could be worth analyzing the wrong predictions in view of identifying the factors that should be taken into account to make HoTMuSiC even more performing.

**Methods**

**Set of experimentally characterized mutations**

We started collecting the mutations with experimentally measured $\Delta T_m$ value from the ProTherm database [59] and the literature. Each entry was then manually checked from the original literature to remove errors and select those that satisfy the following criteria: were only considered (1) mutations in monomeric proteins of known X-ray structure with resolution below 2.5 Å, (2) mutations whose experimental $\Delta T_m$ was measured in absence of chemical denaturants, (3) simple two-state (un)folding transitions, and (4) single point
mutations. Destabilizing or stabilizing mutations by more than 20 °C were overlooked, as they probably induce important structural modifications that our method is unable to model. Using this procedure, we obtained a set $S_{\text{mut}}$ of 1,626 mutations that belong to 90 proteins and have an experimental $\Delta T_m$. More information and their list can be found in [60].

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**Author contributions statement**

F.P. and M.R. conceived the experiment(s), F.P. and R.B. conducted the experiment(s), F.P. and M.R. analyzed the results, F.P. and M.R. wrote the paper. All authors reviewed the manuscript.

**Additional information**

The authors declare no competing financial interests.

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