Correction

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Multiensemble Markov models of molecular thermodynamics and kinetics

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We introduce the general transition-based reweighting analysis method (TRAM), a statistically optimal approach to integrate both unbiased and biased molecular dynamics simulations, such as umbrella sampling or replica exchange. TRAM estimates a multiensemble Markov model (MEMM) with full thermodynamic and kinetic information at all ensembles. The approach combines the benefits of Markov state models—clustering of high-dimensional spaces and modeling of complex many-state systems—with those of the multistate Bennett acceptance ratio of exploiting biased or high-temperature ensembles to accelerate rare-event sampling. TRAM does not depend on any rate model in addition to the widely used Markov state model approximation, but uses only fundamental relations such as detailed balance and binless reweighting of configurations between ensembles. Previous methods, including the multistate Bennett acceptance ratio, discrete TRAM, and Markov state models are special cases and can be derived from the TRAM equations. TRAM is demonstrated by efficiently computing MEMMs in cases where other estimators break down, including the full thermodynamics and rare-event kinetics from high-dimensional simulation data of an all-atom protein–ligand binding model.

Significance

Molecular dynamics simulations can provide mechanistic understanding of biomolecular processes. However, direct simulation of slow transitions such as protein conformational transitions or protein–ligand dissociation are unfeasible with commonly available computational resources. Two typical strategies are (i) conducting large ensembles of short simulations and estimating the long-term kinetics with a Markov state model, and (ii) speeding up rare events by bias potentials or higher temperatures and estimating the unbiased thermodynamics with reweighting estimators. In this work, we introduce the transition-based reweighting analysis method (TRAM), a statistically optimal approach that combines the best of both worlds and estimates a multiensemble Markov model (MEMM) with full thermodynamic and kinetic information at all simulated ensembles.

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Let us consider a molecular system in a reference ensemble, given the simulation data at all temperatures and pressures. The system has an equilibrium distribution \( \mu(x) \) of the ensemble that contains \( k \) different ensembles (indexed by the superscript \( k \)), which may comprise an arbitrary combination of the unbiased ensemble, simulations with biased energy functions, or different temperatures. We can formally relate any ensemble with dimensionless potential \( f^k(x) \) to the reference ensemble by introducing a bias potential \( b^k(x) \) such that \( \mu^k(x) = u(x) + b^k(x) \). The corresponding equilibrium distribution \( \mu^k(x) \) of the \( k \)th ensemble can be expressed as follows:

\[
\mu^k(x) = e^{f^k(x) -\beta b^k(x)} \mu(x),
\]

where the relative free energy \( f^k \) of ensemble \( k \) is chosen such that \( \mu^k(x) \) is normalized. Consider the following examples to see how \( b^k(x) \) must be chosen to model commonly used enhanced sampling methods:

1) In US, the potential energy function of each simulation is \( U(x) + B^k(x) \), where \( B^k(x) \) is the \( k \)th umbrella potential. The bias potential is as follows:

\[
b^k(x) = \beta B^k(x).
\]

2) Replica exchange or parallel tempering simulations are performed at different temperatures \( T^1, \ldots, T^K \), the bias of the \( k \)th temperature with respect to the reference ensemble (e.g., the lowest temperature) is as follows:

\[
b^k(x) = U(x)(\beta^k - \beta).
\]

**MSMs for Molecular Kinetics.** An MSM at ensemble \( k \) consists of a partition of the molecular configuration space into \( m \) discrete and nonoverlapping configuration states \( S_1, \ldots, S_m \) and the conditional transition probabilities \( p^k_{ij}(\tau) \) that a system that is in state \( S_i \) at time \( t \) will be found in state \( S_j \) at time \( t + \tau \).

We first define the local free energy \( f^k \) of configuration state \( S_i \) in ensemble \( k \). The exponential of \( f^k \) is proportional to the statistical weight of this state:

\[
e^{-f^k} = e^{-f^k} \int_{S^k} \mu^k(x) dx
\]

where the integral evaluates to the equilibrium probability of the system to be in state \( S_i \) when simulated in ensemble \( k \).

For given simulation data from ensemble \( k \) that contains \( c^k_{ij} \) transitions from state \( S_i \) at time \( t \) and to state \( S_j \) at time \( t + \tau \), the likelihood of an MSM with transition matrix \( p^k = \{ p^k_{ij} \} \) is as follows:

\[
L^k_{\text{MSS}} = \prod_{i=1}^{m} \prod_{j=1}^{m} (p^k_{ij})^{c^k_{ij}}.
\]

When simulations are conducted at thermal equilibrium (i.e., without adding or removing energy to the system) in ensemble \( k \), equilibrium and transition probabilities are related by the detailed balance equations \( e^{-\beta} p^k_{ij} = e^{-\beta} p^k_{ji} \), and the Markov model is said to be reversible. With detailed balance constraints, the maximum likelihood of Eq. 6 has no closed-form solution but can be iteratively solved (28, 62, 63).

**Local Equilibrium Model.** If simulations sample from multiple ensembles, a central problem is to infer the equilibrium distribution \( \mu(x) \) at a reference ensemble, given the simulation data at all ensembles. The principle behind such inference is that we can reweight the equilibrium probability of a sample \( x \) between different ensembles by means of Eq. 2.

A widely used estimator is the binless WHAM method, (50, 51), also called MBAR (49), which provides an optimal estimate of \( \mu(x) \) under the assumption that at each ensemble \( k \), the samples \( x \) are drawn independently from their global equilibrium distribution \( \mu^k(x) \). MBAR can be derived by maximizing a likelihood that is simply given by the product of \( \mu^k(x) \) over all samples \( x \) and all ensembles \( k \) (49–51).
However, we do not want to depend on the global equilibrium assumption. Hence we define the local equilibrium distribution for each configuration state $S_i$:

$$
\mu_i^k(x) = \begin{cases} 
\exp^{-\beta k} \mu_i^k(x) & x \in S_i \\
0 & \text{else},
\end{cases}
$$

[7]

We assume that simulations are sampling from these local equilibrium distributions, but they do not need to be in equilibrium between configuration states, which is key for invoking the MSM framework. We obtain the following likelihood of generating the simulation data for a given sequence of discrete states:

$$
L_{\text{LEQ}}^k = \prod_{i=1}^m \prod_{x \in X_i} \mu_i^k(x),
$$

[8]

where $X_i^k$ denotes the set of all samples generated from the $k$th ensemble and in configuration state $S_i$. As $\mu_i^k(x)$ can be related to $\mu(x)$ via Eqs. 2 and 7, the local equilibrium model is key to reweight samples between different ensembles.

**TRAM Likelihood.** We develop the TRAM estimator. The TRAM likelihood combines the MSM likelihood [6] and local equilibrium likelihood [8]. Inserting Eqs. 2 and 7, we obtain the following:

$$
L_{\text{TRAM}} = \prod_{k=1}^K \left( \prod_{i,j} \left( p_{ij}^k \right)^{c_{ij}^k} \right) \left( \prod_{m=1}^m \prod_{x \in X_i} \mu(x) \exp^{-\beta v_m^k(x)} \right) \left( L_{\text{MSE}}^k \right) L_{\text{LEQ}}^k.
$$

[9]

This likelihood expresses the probability that a given set of trajectories sampling from different ensembles has visited a particular sequence of discrete states ($L_{\text{MSE}}^k$) and has sampled the local configurations inside these discrete states ($L_{\text{LEQ}}^k$). The structure of the TRAM likelihood is similar to that of a hidden Markov model (64).

The trajectory statistics include the bias potentials $b_i^k(x)$ that are defined by the simulation protocol [e.g., US or replica exchange molecular dynamics (REMD)], and the number of observed transitions $c_{ij}^k$. The unknown variables in the TRAM likelihood are the point densities $\mu(x)$, the local free energies $f_i^k$, and the transition probabilities $p_{ij}^k$. The TRAM problem is to maximize the likelihood [9] in the variable space subject to the following constraints:

$$
\exp^{-\beta f_i^k} p_{ij}^k = \exp^{-\beta f_j^k} p_{ji}^k, \quad \text{for all } i,j,k
$$

[10]

$$
\sum_j p_{ij}^k = 1, \quad \text{for all } i,k
$$

[11]

$$
\sum_{x \in X} \mu(x) = 1,
$$

[12]

where [11] and [12] are simple normalization constraints, and $\mu(x)$ is considered as a discrete distribution on the set of all samples, $X$. The detailed balance condition [10] couples the dynamical (MSM) part to the local equilibrium part. Unfortunately, the detailed balance constraints make the above problem very hard to solve.

**Maximum-Likelihood Solution.** The TRAM problem contains $(m^2K + |X|)$ unknowns, $(mK + 1)$ linear equality constraints (normalization), and $Km(m - 1)/2$ nonlinear equality constraints (detailed balance), so finding the optimal solution by directly using gradient- or Newton-type methods is difficult even for systems with only few configuration states or ensembles. Fortunately, we can transform the TRAM problem into a more tractable system of nonlinear algebraic equations and solve the resulting system by an iterative algorithm. By using the Lagrange duality theory (Appendix), it can be proved that the maximum of the TRAM likelihood satisfies the following equations:

$$
\sum_i \exp(f_i^k - b_i^k) = 1, \quad \text{for all } i,k
$$

[13]

$$
\sum_i \exp(f_i^k - b_i^k) = 1, \quad \text{for all } i,k
$$

[14]

where $v_i^k$ are Lagrange multipliers that can be interpreted as counts (for infinite statistics $v_i^k = \sum c_{ij}^k$, see Appendix). $X_i$ is the set of all samples in configuration state $S_i$, no matter from which ensemble. The factor $R_i^k$ is given by the following:

$$
R_i^k = \sum_j \left( \frac{c_{ij}^k + c_{ji}^k}{\exp(f_j^k - f_i^k)v_j^k + v_i^k} \right),
$$

[15]

where $N_i^k$ is the number of samples in $X_i^k$. $R_i^k$ are effective state counts (see below). When [9–12] are fulfilled and in the limit of infinite statistics, $R_i^k = N_i^k$.

In contrast to Eqs. 9–12, the formulation in [13] and [14] only contains the $2mK$ unknowns $v_i^k$ and $f_i^k$ and does not involve $p_{ij}^k$ and $\mu(x)$ explicitly. Given the solution of Eqs. 13 and 14, we can compute all MSM transition matrices by the following:

$$
p_{ij}^k = \frac{c_{ij}^k + c_{ji}^k}{\exp(f_j^k - f_i^k)v_j^k + v_i^k},
$$

[16]

and the unbiased statistical weights of all samples by the following:

$$
\mu(x) = \frac{1}{\sum_i R_i^k \exp(f_i^k - b_i^k)},
$$

[17]

where we have defined $i(x)$ such that $i(x) = j$ when $x \in X_j$. The TRAM estimator defined by Eqs. 13 and 14 is statistically optimal, asymptotically correct, i.e., converges to the correct results of $f_i^k$, $p_{ij}^k$, and $\mu(x)$ as the length or number of simulation trajectories increases, and the most general multiensemble Markov model estimator (see below and Appendix).

Eqs. 13 and 14 are reminiscent of other estimators: Eq. 13 arises when optimizing an MSM transition matrix with given stationary weights $\exp(-f_i^k)$ (63). Eq. 14 has the same form as the self-consistent MBAR equation (49) for the ensemble free energies $f_i^k$ of a single configuration state $S_i$, but instead of the number of samples in that state $N_s$, the modified counts $R_i^k$ are used (detailed interpretation in Appendix). The TRAM equations can therefore be thought of expressing two optimization problems simultaneously: (i) at each ensemble $k$, the optimization of the MSMs for given free energies, $f_i^k$ for all configurations $S_i$. (ii) At each configuration $S_i$, the optimization of the free energies, $f_i^k$ for all ensembles.

**Optimization Algorithm.** The TRAM equations [13] and [14] are coupled and can only be solved numerically. Here, we transform
them into a simple fixed-point problem, in which the following equations need to be iterated until convergence:

$$v_i^{k, \text{new}} := v_i^k - \sum_j \frac{c_{ij}^k}{\exp\left(\frac{f_j^k - f_i^k}{k_B T}\right)} v_j^k + v_i^k \quad [18]$$

$$f_i^{k, \text{new}} := -\ln \sum_{x \in X} \sum_{R^j} \exp\left[f_j - b^k(x)\right] \quad [19]$$

More implementation details of this algorithm, including initialization, termination, and convergence acceleration are given in Appendix. Note that, instead of a fixed-point iteration, we could attempt a Newton-based (65, 66) or stochastic optimization method (67, 68).

**Thermodynamics and Kinetics from TRAM.**

**Thermodynamics.** The correct calculation of stationary (thermodynamic) properties does not rely on Markovianity, but only requires the unbiased estimation of free energies $f_i^k$ or the stationary density $\rho_i^k(k)$ at the chosen lag time $\tau$. However, it is required that the simulations are in local equilibrium within the configuration states, and violations of local equilibrium can be compensated by using longer lag times $\tau$. The robustness of TRAM estimates should therefore be tested as a function of lag time (see results, Fig. 3).

**Kinetics.** Asymptotic correctness of all $f_i^k$ at the selected lag time $\tau$ does not imply that powers of the matrix $P^k$ are a good prediction of the transition probabilities at longer lag times. Whether the multiensemble Markov model is able to predict long-term kinetics depends on the quality of the discretization and on $\tau$ being sufficiently large, as usual for MSMs (28, 56) (see results, Fig. 3D). Note that this behavior does not change if a rate matrix is used instead of a transition matrix.

**Generality of TRAM.** TRAM is a generalization of discrete TRAM, binless WHAM/MBAR, binned WHAM, and reversible MSMs (Fig. 2). These specialized estimators can be derived from TRAM by adding the specific assumptions made by them. MBAR can be derived from TRAM by assuming that samples are drawn from the global equilibrium distribution of each ensemble. Discrete TRAM can be derived by assuming that the bias energies are piecewise constant and pointwise reweighting can be replaced by histogram reweighting. WHAM is derived using a combination of both assumptions. Finally, if we have only a single ensemble, the TRAM solution is identical to the reversible MSM estimator (derivations in Appendix).

TRAM has the Markovianity assumption in its likelihood model, but otherwise only uses fundamental relations such as detailed balance and pointwise reweighting. It is therefore the most general MSM-based estimator for simulation data from multiple ensembles. Other transition-based reweighting methods are related as follows: trajectory reweighting techniques (58–60) are applicable without any state space discretization, but assume the trajectory starting points to emerge from a global equilibrium distribution. The dynamic weighted histogram analysis method (DHAM) (57) uses a kinetic reweighting scheme that can predict kinetics at ensembles not simulated from, but is based on a rate model, uses histogram binning and does not optimize with respect to detailed balance. An advantage in not enforcing the detailed balance constraint is that DHAM is a single-shot estimator, while TRAM and dTRAM are estimators that need to be iterated to solution. xTRAM (55) is a bin-less TRAM method, but in contrast to the present method not statistically optimal for finite data (*Applications*).

**Applications**

**Multitemperature Replica-Exchange.** We compare the performance of MBAR and TRAM for REMD simulations of solvated alanine dipeptide using 33 exponentially spaced temperatures in the range of 300–600 K (Fig. 34) (see ref. 55 for simulation protocol). In multitemperature simulations, the bias potentials between ensembles depend on the potential energy (Eq. 4). To analyze such data with histogram-based methods, one would have to bin the potential energy axis in addition to the coordinate(s) of interest (69). For many-body systems such as solvated macromolecules, the large range of potential energies sampled and the required resolution to approximate the bias energies (Eq. 4) disables bin estimators such as WHAM, discrete TRAM, and DHAM, and instead require binless methods such as MBAR and TRAM.

The configuration space of alanine dipeptide is partitioned into 20 discrete states using $k$-means clustering in the space of the coordinates ($\cos \phi, \sin \phi, \cos \psi, \sin \psi$) (Fig. 3A). The equilibrium probabilities on the sets I–IV are compared between estimators. Even after this relaxation phase, TRAM effectively and efficiently converges to the same values, whereas TRAM converges significantly faster (Fig. 3B). TRAM outperforms MBAR because TRAM relies only on local rather than global equilibria. As a result, TRAM does not suffer from the fact that initial structures are not sampled from a global equilibrium distribution, and the REMD simulation must first relax to sample from global equilibrium. Even after this relaxation phase, TRAM effectively and efficiently converges to the same values, whereas TRAM converges significantly faster (Fig. 3B). TRAM outperforms MBAR because TRAM relies only on local rather than global equilibria. As a result, TRAM does not suffer from the fact that initial structures are not sampled from a global equilibrium distribution, and the REMD simulation must first relax to sample from global equilibrium. Even after this relaxation phase, TRAM effectively and efficiently converges to the same values, whereas TRAM converges significantly faster (Fig. 3B).

Next, we test the robustness of TRAM estimates as a function of the lag time (Fig. 3 C and D). It is seen that the stationary probabilities, and thus the results in Fig. 3B, are independent of the lag time, demonstrating that the Markov property is not required to get correct estimates of the stationary properties. Unbiased estimates of equilibrium properties only require that the simulations are in local equilibrium. For REMD simulations with a good state space discretization, this is fulfilled even at short lag times $\tau$. In contrast, unbiased estimates of the kinetic properties require sufficiently long lag times for the Markov property to be valid. The estimated relaxation timescale at temperature 366 K is constant above $\tau = 10$ ps (Fig. 3D), which was used for all TRAM estimates in Fig. 3. Only trajectory segments in which no temperature swap was executed for 10 ps or longer were used for this estimate.

Fig. 3 E and F show thermodynamics and kinetics obtained from the multiensemble Markov model as a function of the temperature. The probabilities of metastable states become more similar with increasing temperatures, but the temperature dependence is very weak, indicating that entropy differences play a minor role (Fig. 3E). The mean first passage times (inverse transition rates) from I/II to III/IV and back decrease strongly with temperature (Fig. 3F). The decrease is exponential (Arrhenius-like) up to 450 K, but shows a weaker temperature dependence.
Comparison of MBAR, xTRAM, and TRAM for estimating the equilibrium distribution of a three-well potential from US data. (A) Potential function $u(x,y)$, where thin white lines represent the borders of 20 discrete states, thick white lines represent the borders of the three potential wells, and the dashed black lines indicate the umbrella centers. (B) A simulation trajectory with the bias potential centered at $x = 8.33$. (C) Autocorrelation times $\tau_{\text{ess}}(x)$ and $\tau_{\text{ess}}(y)$ with respect to $x$ axis and $y$ axis for different umbrella centers. (D) Average estimation errors and their SDs of MBAR, xTRAM, and TRAM for different simulation trajectory lengths over 30 independent realizations of US.

Biased Simulations with Slow Orthogonal Degrees of Freedom. Simulations in which sampling is enhanced along predefined reaction coordinates (e.g., using bias potentials) are often hampered by unforeseen rare transitions in other coordinates (52). For illustration, we use a pathological 2D toy potential with three wells (Fig. 4A). US simulations are conducted using only the $x$ coordinate as bias coordinate (details in Appendix).

As the potential wells I and III cannot be separated on the $x$ axis, it takes a long time to converge to the global equilibrium when the simulations are confined to values of $x < 15$. Especially simulations with the second umbrella potential centered at $x = 8.33$ exhibit rare-event transitions along the $y$ axis (Fig. 4B), characterized by an autocorrelation time of 500 steps (Fig. 4C) (70). In contrast, the largest value of the autocorrelation time along the $x$ axis is only about 22 steps.

Rare events in nonenhanced coordinates are a common problem in enhanced sampling simulations and cause major problems in their analysis. For estimation methods relying on global equilibrium sampling such as MBAR, the statistically correlated samples should be discarded before running the estimator (49). In umbrella simulation 2, this would result in retaining only one effective sample for each 500 samples in the simulation, resulting in the loss of almost all data and requiring very long simulation times.

TRAM does not require global equilibrium sampling and can therefore use simulation data much more efficiently. We discretize the configuration space $\Omega$ into 20 states as shown in Fig. 4A, and then use TRAM with lag time $\tau = 1$ to estimate the unbiased equilibrium distribution from the US data. Fig. 4D summarizes estimation errors of TRAM for different lengths of each simulation trajectory and compares them with those of MBAR and the previously described xTRAM method (55). Here, the error is evaluated as the Kullback–Leibler divergence between the estimated probability distributions of the three macrostates I, II, and III and the true reference. In contrast to MBAR, TRAM can effectively overcome the influence of the nonequilibrium distribution of the data through Markov state modeling and achieve accurate estimates even in the case of trajectory length smaller than autocorrelation times $\tau_{\text{ess}}(y)$ of some biased simulations. Furthermore, TRAM also significantly outperforms xTRAM, which is a consistent estimator under the MSM assumption but not statistically optimal for finite data.

### Protein–Ligand Binding and Kinetics

Finally, we demonstrate that TRAM can help to resolve the problem of rare events in orthogonal degrees of freedom and provides efficient estimates of rare-event kinetics in all-atom, explicit-solvent simulations of the serine protease trypsin and its inhibitor benzamidine (see ref. 34 for detailed setup). This illustrates the usefulness of the estimator.
in high-dimensional spaces where binning of all relevant coordinates is not an option.

We first analyze pure US simulations with 150 umbrella windows used to sample the position of benzamidine between the bound pose and a prebinding site (Fig. 5A, structures i–iv; details in Appendix). To detect rare events in the unbiased coordinates, time-lagged independent component analysis (TICA) (29, 30) was used with the Cartesian coordinates of residues around the binding site. The first independent component (IC) is strongly correlated with the US coordinate. From the remaining ICs, two had timescales implied by the TICA eigenvalues larger than the trajectory length, indicating undesirable metastable transitions orthogonal to the umbrella coordinate. The second IC corresponds to closing of the binding pocket by the Trp 215 side chain (Fig. 5D, structures i and iii). The third IC corresponds to an isomerization of the disulfide bond between Cys 191 and Cys 220. An analysis using MBAR or WHAM is thus unfeasible or inefficient, as the global equilibrium assumption is strongly violated.

One strategy to deal with this very common problem is to re- strain coordinates orthogonal to the umbrella coordinate, to avoid undesirable degrees of freedom from switching (45). Although this approach is useful for computing energy differences between end states, it may change or restrain the transition mechanism and artificially increase free-energy barriers along the pathway. With TICA and TRAM, we now have the possibility to allow these orthogonal dynamics to happen, and to treat these events explicitly.

The space spanned by the US coordinate and the second IC was discretized into 100 Voronoi cells with the k-means algorithm (Fig. 5A). This number of states is far smaller than the number of bins that would be required with a binned estimator such as WHAM or discrete TRAM. A count matrix $C^D$ was estimated for every umbrella at a lag time of 11 ns, and the largest strongly connected component $\mathcal{S}$ of the summed count matrix $C^D = \sum C^d$ was determined. The initial set was strongly disconnected, and we therefore adaptively started new umbrella simulations in nine rounds, to improve the connectivity (Appendix). In the complete dataset, some clusters are still disconnected (red clusters in Fig. 5A).

In particular, these disconnected states include structures in which the binding site is occluded by a tryptophan side chain, while benzamidine is still inside, and structures in which the binding site attempts to close during the exit pathway. TRAM is applied on the connected subset of states (white clusters in Fig. 5A). The TRAM results show that the Trp-occluded conformation is a local minimum in the free-energy landscape (Fig. 5C). This is confirmed by refs. 13 and 61 where the Trp-occluded conformation is shown to be a metastable conformation of the protein. In contrast, this local minimum is not found by MBAR, and several disconnected minima are spuriously estimated (boxes in Fig. 5B).

To analyze the full high-dimensional binding mechanism and estimate unbiased kinetics, we must go beyond US simulations. We therefore used TRAM to combine the US data with up to 49,1 μs of unbiased MD data (details in Appendix). The unbiased trajectories started in the unbound state, such that many binding events spanned the US coordinate and the second IC.

**Fig. 5.** Thermodynamics and kinetics of all-atom protein-ligand binding model for trypsin-benzamidine. (A–C) US simulations. (D and E) MEMM using both unbiased and US simulations. (A) Trajectories projected on the space of the umbrella sampling (US) coordinate and the second independent component (IC). The US coordinate describes a transition from benzamidine bound to Asp-189 to benzamidine located outside the binding pocket on the surface of trypsin. The second IC corresponds to concerted opening of loop (Trp-215-Gln-221) and flipping of Trp-215. The Voronoi centers of the Markov states are shown as disks. Markov states that are irreversibly connected to the data set are shown as red disks and are excluded from the MEMM. (B) Potential of mean force (PMF) in the space spanned by the US coordinate and the second IC. The space spanned by the US coordinate and the second IC was discretized into 100 Voronoi cells with the k-means algorithm (Fig. 5A). This number of states is far smaller than the number of bins that would be required with a binned estimator such as WHAM or discrete TRAM. A count matrix $C^D$ was estimated for every umbrella at a lag time of 11 ns, and the largest strongly connected component $\mathcal{S}$ of the summed count matrix $C^D = \sum C^d$ was determined. The initial set was strongly disconnected, and we therefore adaptively started new umbrella simulations in nine rounds, to improve the connectivity (Appendix). In the complete dataset, some clusters are still disconnected (red clusters in Fig. 5A).

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To analyze the full high-dimensional binding mechanism and estimate unbiased kinetics, we must go beyond US simulations. We therefore used TRAM to combine the US data with up to 49,1 μs of unbiased MD data (details in Appendix). The unbiased trajectories started in the unbound state, such that many binding
events are present. Individual steps of dissociation events are found in some single trajectory, but no complete dissociation event is found in any single trajectory. By combining the free-energy information inherent in the biased trajectories with the binding kinetics from the unbiased trajectories, the full unbinding kinetics can be estimated with TRAM. TRAM gives the estimate $k_{\text{eff}}^{\text{TRAM}} = 1.170 \text{ s}^{-1}$, with 95% confidence intervals of $[0.67, 2.120 \text{ s}^{-1}]$. For comparison, the MSM estimated from the unbiased simulation data only, using the same definition of states and lag time as for TRAM, provides an estimate of $k_{\text{eff}}^{\text{MSM}} = 1.863 \text{ s}^{-1}$ with a larger uncertainty of $[0.867, 4.816 \text{ s}^{-1}]$ [all errors estimated using bootstrap, experimental dissociation rate $600 \text{ s}^{-1}$ (71)].

To assess the data efficiency of TRAM and the MSM, we varied the amount of unbiased MD data that was used for the estimation. With TRAM, only 5–10% of the unbiased MD data are needed compared with an MSM to reliably estimate $k_{\text{eff}}$ (Fig. 5E).

Fig. 5D shows a kinetic network of the binding/dissociation events at the unbiased ensemble of the multiensemble Markov model. The kinetic data include association to several secondary binding sites; two of them are shown in Fig. 5D. At the lag time that we chose (30 ns), the prebound states $ii$ and $iv$ are not metastable and indirect transitions where these states are skipped during binding/unbinding appear in the transition matrix (not shown in the figure for clarity).

Conclusions

We have derived the TRAM for estimating MEMMs from simulation data comprising arbitrary combinations of unbiased MD, biased enhanced sampling simulations such as US, or multitemperature simulations such as REMD. TRAM does not require binning of the bias energies and is therefore suitable for the analysis of multitemperature simulations and of high-dimensional state spaces. TRAM is a Markov modeling method as it only requires local equilibrium and uses conditional transition statistics to estimate the MEMM—i.e., it can use short trajectories whose starting points were not sampled from global equilibrium. Even when just being used for estimating thermodynamics, e.g., the equilibrium distribution at the unbiased ensemble, or temperature-dependent free energies, TRAM is superior to global equilibrium-based estimators such as WHAM or MBAR.

In an application to US simulations of protein–ligand binding, we have used MSM concepts of finding slow coordinates and detecting a connected set of states to define a meaningful subspace for computing a ligand dissociation pathway and its free-energy profile. We have also sketched an approach to identify sampling bottlenecks and extend simulations in nonconverged umbrella windows to adaptively improve the convergence of the umbrella simulation, in line with other adaptive approaches (52, 72). In this example, combining US with replica exchange simulations may also have improved the sampling (73, 74).

We demonstrated that TRAM can be used to compute an unbiased estimate of protein–ligand dissociation kinetics on the order of a millisecond by using only a few microseconds of simulation data. Beyond the simple two-state rate, TRAM is ideally suited to estimate the full multistate kinetics that was found in refs. 13 and 46 with rate models or much more simulation data. TRAM significantly expands the power of the MSM framework by allowing to integrate the full power of enhanced sampling simulations. The TRAM estimator is included in PyEMMA (75) as of version 2.2. Tutorials can be found under Tutorials can be found under "Trapping in Ttract". The TRAM estimator is included in PyEMMA (75) as of version 2.2. Tutorials can be found under Tutorials can be found under "Trapping in Ttract".

Materials and Methods

Three-Well Potential Set. The potential shown in Fig. 4A is defined by a sum of four Gaussians $u(x,y) = -\sum_{i=1}^{4} g_{i}(x_{b}, h_{i}, r_{c}, r_{w})(x,y)$ with parameters $(8,15,15,10,10), (4.8,9,9,2.5,2.5), (8,9,2,1.2,2.5,2.5)$, and $(4.2,13,2.5,2.5)$, and $g_{i}(x_{b}, h_{i}, r_{c}, r_{w})(x,y) = \exp(-((x-h_{i})^{2}/r_{w}^{2}) - ((y-h_{i})^{2}/r_{w}^{2}))$ on a square $[5,25] \times [5,25]$ and outside US. MD simulations are conducted using bias potentials $b^{k}(x,y) = (x-x_{w})^{2}/5$ for $k = 1, \ldots , 7$ with umbrella centers $x_{w}$ positioned at $x_{w} = [10k+5]/3$. We generate 20 independent simulation trajectories $(\{x_{i}^{k}, y_{i}^{k}\})$ for each biased potential using the Metropolis sampling algorithm, where $x_{i}^{k}, y_{i}^{k}$ is randomly drawn from $[5,25]$ and the candidate sample follows the uniform distribution on $[x_{w} - 3, x_{w} + 3 \times [y_{w} - 3, y_{w} + 3)$ for a given $(x_{w}, y_{w})$. The autocorrelation time of $(\{x_{i}^{k}, y_{i}^{k}\})$ in $x$ is given by $\tau_{a} = 1 + 2\Sigma \tau_{i}$, and likewise in y (70), where $\rho_{i}$ denotes the autocorrelation at lag $s$ of $h$. We compute $\tau_{a}$ from a long trajectory with 100 steps as described in ref. 76.

Trypsin–Benzamidine Setup.

US. The US coordinate is defined as the distance from the center of mass of all backbone atoms of Asp 189 and Pro 161 to the center of mass of the benzamidine ring atoms. The setup consists of 150 harmonic umbrellas with uniform force constant of 100 kcal mol$^{-1}$ Å$^{-2}$ and umbrella centers positioned along the US coordinate according to $x_{\text{US}} = 10.5 \text{ Å}$ for $i = 0$ … 149. For each umbrella, multiple independent runs were generated all starting from the same initial conditions that come from an unbiased binding trajectory. In total, 459 trajectories each having a length of 20 ns were generated adaptively in nine rounds of restarts. After an initial exploratory round, eight additional rounds were started to increase the overlap $\langle \Sigma \text{min}M_{i} \rangle / \langle \text{mean}M_{i} \rangle$ between ensembles. Restarts were done in ensembles $k = 1$ where $\rho_{k} < 100$. For the analysis, TICA (29) was used at a lag time 5.5 ns on the Cartesian coordinates of all heavy atoms within a 15 Å radius around Asp 189 in the Protein Data Bank structure.

Molecular dynamics. A total of 491 unbiased MD simulation trajectories of length 100 ns each (data from ref. 34) were discretized by selecting the nearest neighbor heavy-atom contacts between benzamidine and all trypsin residues as input features (75). The features were then used for the TICA (lag time, 5 ns) to a kinetic map preserving 95% of the kinetic variance (31), resulting in a 31-dimensional transformed space. Discretization of all data in the joint space of 31 ICs and the two coordinates shown in Fig. 5A was done with the k-means algorithm, using $k = 500$. Microstates were grouped into seven macrostates. Four macrostates correspond to the quadrants of Fig. 5A, splitting microstates near the binding site at the US coordinate 14.5 Å and the TICA coordinate $g = 1$. Nearness to the binding site is defined by an US coordinate $x_{\text{US}} < 18.2 \text{ Å}$ and being inside a binding funnel, defined by $\cos \gamma < 0.74$, where $\gamma$ measures the angle between the vectors connecting centers of mass of benzamidine with Pro 161 and Trp 215 with Pro 161. The remaining microstates were grouped in three macrostates: the unbound state and two alternatively bound states where benzamidine binds to secondary binding sites of trypsin, found with PCCA++ (77).

Kinetics. Using TRAM, MEMMs were estimated combining the US data and the unbiased MD data. The MEMM lag times were chosen as 30 ns for the unbiased data and as 10 ns for the US data (chosen from the interval where $k_{\text{eff}}$ appears to be independent of both lag times). A transition matrix for the unbiased ensemble was computed according to Eq. 16. $k_{\text{eff}}$ was computed as the reciprocal of the mean-first-passage time from the bound macrostates (US coordinate < 14.5 Å) to the unbound state.

Bootstrap. Errors bars for the different estimates were obtained from a bootstrap. Every sample of the bootstrap was generated by first partitioning the trajectories by ensemble, and then independently for every partition drawing whole trajectories and finally merging the trajectories.

Appendix

Solution of the TRAM Problem. Ignoring constants, the constrained optimization problem of the TRAM log likelihood [1] can be written as follows:

$$
\min_{\{\rho(x)\}, \{P_{i}\}} \left\{ \sum_{k,i,j} c_{k,i,j} \ln \rho_{k,i}^{*} - \sum_{k,i,j} N_{k,i}^{*} P_{i,j}^{*} - \sum_{x \in X} \ln (\rho(x)) \right\}
$$

s.t. $c_{k,i,j}^{*} P_{i,j}^{*} = c_{k,i,j}^{*} P_{i,j}^{*}$ for all $i$, $j$, $k$

$P_{i,j}^{*} = 1$, for all $i$, $k$

with

$$
\rho_{i}^{*} = -\ln \sum_{x \in X} \rho(x) e^{-h_{i}(x)}.
$$

We omit the normalization constraint [17], because the normalization of $\rho(x)$ does not affect the optimality of the solution of Eq. 20, and we can thus normalize $\rho(x)$ a posteriori.

Wu et al. | PNAS | Published online May 25, 2016 | E3227
Using the Lagrange duality lemma of discrete TRAM problem (56), it can be shown that Eq. 20 is equivalent to the following unconstrained min optimization problem:

$$\min_{\{v^i\}} \mathcal{L}_{\text{dual}} = \sum_{k,i} c^k_i \ln \left( e^{-\mu^k_i} v^k_i + e^{-\mu^k_j} v^j_i \right) - \sum_k \left( N^k - \sum_j c^k_j \right) p^k_i$$

$$- \sum_i v^k_i = \sum_{x} \ln \mu(x),$$

where $v^i = \{v^k_i\}$ are Lagrange multipliers. Equivalence means that the optimal solution of Eq. 20 can be obtained from that of Eq. 22 by using Eq. 16.

We now consider solving Eq. 22. Because $\mathcal{L}_{\text{dual}}$ is a concave function of $\mathcal{C}^d$ and a convex function of $\{\ln \mu(x)\}$, the optimal solution of Eq. 22 can be characterized as a saddle point with $\partial \mathcal{L}_{\text{dual}} \partial v^k = 0$ and $\partial \mathcal{L}_{\text{dual}} \partial \mu = 0$ for all $i, k$, and $x$ (see section 10.3.4 in ref. 78). Because

$$\frac{\partial \mathcal{L}_{\text{dual}}}{\partial \mu} = \sum_i \left( \frac{e^{\mu^k_i}}{\mu^k_i} - 1 \right),$$

$$\frac{\partial \mathcal{L}_{\text{dual}}}{\partial v^k} = \sum_i \left( \frac{e^{\mu^k_i}}{\mu^k_i} - \mu^k_i \right),$$

where $R^k_i$ is defined in Eq. 15, we can conclude that the optimal solution of Eq. 22 should satisfy Eq. 13, and Eq. 17 holds. Substituting Eq. 17 into Eq. 21, we can get the optimality condition [14].

Asymptotic Correctness of TRAM. We use $\bar{B}$ to denote the exact value of an unknown variable $b$ without any statistical error, and denote by $c^k_i = \sum_i c^k_i$ the sum of row $i$ in count matrix $C^k = [c^k_i]$, and by $N_i$ the number of samples in $S_i$ ($c^k_i$ is different from $N^k_i$ for finite statistics).

Now we show that the TRAM estimates of local partition functions, transition matrices, and reference distribution converge to the correct ones under the condition that the size of simulation data (either length of simulation trajectories) tends to infinity and the ratio $N^k_i / N_i$ tends to a constant $w^k_i$ for any $i, k$ under the assumption that the local equilibrium within each configuration $S_i$ is achieved in simulations. In this limit, the transition counts become the following:

$$c^k_i = c^k_i \hat{p}^k_i,$$

Substituting Eq. 25 into Eqs. 16, 15, and 13, and replacing $f^k_i, v^k_i$ with $f^k_i, c^k_i$, we have $p^k_i = \hat{p}^k_i$, $\hat{R}^k_i = N^k_i$, and

$$\sum_i \frac{c^k_i + c^j_i}{f^k_i - f^j_i} = \sum_i \hat{p}^k_i = 1.$$

Define the average equilibrium distribution within $S_i$ over multiple ensembles as $\pi(x) = \sum_i w^k_i \pi^k_i(x)$. According to the law of large numbers, we can get

$$\frac{1}{N_i} \sum_{x} a(x) = \int a(x) \pi(x) dx$$

in the statistical limit for an arbitrary function $a(x)$. According to the above equation, we have the following:

$$\sum_{x \in X \cap S_i} \exp \left[ f^k_i - b^k_i(x) \right] - \exp \left[ f^j_i - b^k_i(x) \right] = \frac{1}{N_i} \sum_{x \in X \cap S_i} \sum_j w^j_i \exp \left[ f^j_i - b^j_i(x) \right]$$

$$= \int \exp \left[ f^k_i - b^k_i(x) \right] \left( \sum_j w^j_i \pi^j_i(x) \right) dx$$

$$= \int \exp \left[ f^k_i - b^k_i(x) \right] \pi(x) dx = 1.$$

From the above, we can conclude that in the statistical limit, the TRAM iterative algorithm converges to $v^k_i = c^k_i$ and $f^k_i = \hat{f}^k_i$, and the estimates of $p^k_i$ given by Eq. 16 are also equal to $\hat{p}^k_i$ in the limit. Moreover, the corresponding estimated reference distribution is as follows:

$$\mu(x) = \frac{1}{\sum_i N^k_i \exp \left[ f^k_i - b^k_i(x) \right]} \pi(x)$$

and it satisfies that

$$E[a(x)] = \sum_i \sum_{x \in X \cap S_i} \frac{a(x)}{N^k_i \exp \left[ f^k_i - b^k_i(x) \right]}$$

$$= \sum_i \frac{1}{N_i} \sum_{x \in X \cap S_i} \sum_j w^j_i \exp \left[ f^j_i - b^j_i(x) \right]$$

$$= \int \pi(x) a(x) dx = E[a(x)]$$

for any function $a(x)$ of the system configuration. So the discrete distribution $\mu(x)$ given by the TRAM algorithm is also a consistent estimate of the reference distribution $\pi(x)$.

Proofs That TRAM Is a Generalization of Discrete TRAM, WHAM, MSMs, and MBAR.

MBAR/Runinless WHAM. Suppose that all simulations are in global equilibrium and there is only one configuration state $S_i$ for the whole configuration space, i.e., $S_i = \Omega$. Then we can rewrite the TRAM equations [13] and [14] by dropping all of the subscripts as $v^k = \bar{c}^k$ and

$$\sum_{x \in X \cap S_i} \exp \left[ f^k_i - b^k_i(x) \right] = 1.$$

Eq. 31 is exactly the MBAR estimation equation for free energies $f^k_i$ (49, 51, 66, 79).

Discrete (histogram-based) TRAM. Discrete (histogram-based) TRAM (56) can be expressed in the TRAM nomenclature by using bias potentials $b^k_i(x)$ that are step functions with

$$e^{-b^k_i(x)} \equiv \bar{c}^k, \text{ for } x \in S_i.$$

Then, $\mu(x)$ in Eq. 17 takes a constant value $\mu_i = (\sum_k R^k_i \exp [f^k_i \bar{c}^k])^{-1}$ on $S_i$, yielding the following estimate of the stationary probability of $S_i$ in the unbiased ensemble:

$$\pi_i = N_i \mu_i.$$
Substituting Eqs. 32, 33, and 17 into the TRAM equation [14], we can obtain \( \exp[-f^k_i] = r_i^k \pi_i \) and rewrite Eq. 33 as follows:

\[
\frac{N_i}{\pi_i} = \hat{\pi}_i^{-1} = \sum_{X_j} c_{X_j}^i \pi_j = \frac{1}{\pi_i} \sum_{X_j} c_{X_j}^i + \frac{N_i}{\pi_i} = \sum_{X_j} c_{X_j}^i \pi_j = \sum_{X_j} \left( c_{X_j}^i + c_{X_j}^i \right) \pi_j \pi_i \frac{r_i^k}{r_j^k} \frac{1}{\pi_i}
\]

Eqs. 13 and 34 are identical to the self-consistent equations of discrete TRAM (56) with bias factors \( \hat{\pi}_i \), which means that discrete TRAM is a special case of TRAM and applies if the bias energies can be discretized without error.

**WHAM.** From the discrete TRAM equations [13] and [34], we can further derive the WHAM equations under the assumption that the global equilibrium is achieved with \( p_i^0 = x_i^0 \propto \pi_i \) and \( c_{X_i}^k = \pi_i \sum c_{X_i}^k \) (56).

**MSMs.** If simulations are only performed at one ensemble, the self-consistent equations for reversible maximum-likelihood estimation of MSMs are a special case of discrete TRAM (56).

**Interpretation of the Effective Counts.** \( R_i^k \). Supposing that we apply MBAR only to the samples in a given configuration state \( S_i \), the estimates of the local free energies \( f^k_i \) are given by the following:

\[
\frac{\exp(f^k_i - b^k(X))}{\exp(f^k_i - b^k(X))} = \frac{\sum_{x \in X_i} N_i}{\sum_{x \in X_i} N_i \exp(f^k_i - b^k(X))} = 1.
\]

This equation has the same form as the TRAM Eq. 14 except that \( R_i^k \) is replaced by \( N_i^k \). Thus, we can interpret \( R_i^k \) as counts. By using Eqs. 15 and 16, we find \( R_i^k = N_i^k - \sum c_{X_j}^k + \sum c_{X_j}^k \). \( N_i^k = \sum c_{X_j}^k \) is the number of visits to \( S_i \) in the initial frames of all trajectories. \( \sum c_{X_j}^k \) can be interpreted as the corrected number of incoming transitions to \( S_i \): First, \( \sum c_{X_j}^k \) converges to \( \sum c_{X_j}^k \) in the limit of infinite statistics. Second, the term \( \sum c_{X_j}^k \) in \( R_i^k \) accounts for the first visit to \( S_i \), which cannot be computed from the MSM alone. What remains to be included into \( R_i^k \) is the effective number of visits to \( S_i \) after the first state transition has happened. A suitable candidate would be the number of incoming transitions to \( S_i \). What distinguishes \( \sum c_{X_j}^k \) from \( \sum c_{X_j}^k \) is that the transition matrix is used in the computation of the former. Moreover, although \( \sum c_{X_j}^k \) and \( \sum c_{X_j}^k \) in principle are two independent variables, the quantities \( \sum c_{X_j}^k \) from \( \sum c_{X_j}^k \) can be interpreted as the corrected number of outgoing transitions are linked by the equation \( \sum v_{X_j}^k p_{X_j}^k + v_i^k = \sum c_{X_j}^k + \sum c_{X_j}^k \) (which can be derived from Eq. 16). So both \( \sum v_{X_j}^k p_{X_j}^k \) and \( v_i^k \) are counts that are corrected by the Markov model, which itself fulfills detailed balance.

**Implementation Notes.** In applications of this paper, we initialize the TRAM iteration with \( v_i^k := 1 \) and \( f_i^k := 1 \) as the convergence of TRAM does not seem to depend on the choice of initial point. We terminate the TRAM algorithm when the maximum change in normalized free energies \( \max_a |f_i^k - f_i^{k-1}| < tol \) with tol being a small number (e.g., \( 10^{-10} \)). Considering that the TRAM equations are invariant with respect to a global shift \( f_i^k \rightarrow \alpha + f_i^k \), we perform the normalization after every iteration such that \( \sum \exp[-f_i^k] = 1 \) for the first ensemble \( k = 1 \) to avoid an uncontrolled drift of \( f_i^k \).

The bias factors \( [\exp[-b^k(X)] \) can easily exceed the maximum range of double-precision floating point numbers, so we perform most calculations in log-space to avoid the numerical overflow or underflow. For all summations of the form \( \log \sum \exp[a_i] \), we use the log-sum-exp formula \( \log \sum \exp[a_i] = \alpha + \log \sum \exp[a_i - \alpha] \), where \( \alpha = \max(a_i) \).

In addition, according to our experience, the convergence of the TRAM algorithm can be significantly sped up by adding an extra update step to each iteration that shifts local free energies \( f_i^k \) by \( \delta_i \) as follows:

\[
f_i^{k, new} = f_i^k + \delta_i,
\]

with

\[
\delta_i = \ln \sum_{k,j} \frac{(c_{X_j}^k + c_{X_j}^i) v_j^k}{v_i^k + \exp(f_i^k - f_j^k)} = -\ln \sum_{k,j} c_{X_j}^k.
\]

Note that we can obtain from Eqs. 15 and 14 that

\[
\sum_{k,j} \frac{(c_{X_j}^k + c_{X_j}^i) v_j^k}{v_i^k + \exp(f_i^k - f_j^k)} = \sum_{k,j} R_i^k \cdot \exp(f_i^k - b^k(X)) - \sum_{k,j} N_i^k
\]

\[
= \sum_{k} \frac{\exp(f_i^k - b^k(X))}{\exp(f_i^k - b^k(X))} - \sum_{k} N_i^k = 0.
\]

Hence \( \delta_i = 0 \) is a necessary condition for the TRAM equations, and the update step [36] does not influence the optimality of the limit of the algorithm.

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