

# Scalable Constant pH Molecular Dynamics in GROMACS

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## Abstract

Molecular dynamics (MD) computer simulations are used routinely to compute atomistic trajectories of complex systems. Systems are simulated in various ensembles, depending on the experimental conditions one aims to mimic. While constant energy, temperature, volume, and pressure are rather straightforward to model, pH, which is an equally important parameter in experiments, is more difficult to account for in simulations. Although a constant pH algorithm based on the  $\lambda$ -dynamics approach by Brooks and co-workers was implemented in a fork of the GROMACS molecular dynamics program, uptake has been rather limited, presumably due to the poor scaling of that code with respect to the number of titratable sites. To overcome this limitation, we implemented an alternative scheme for interpolating the Hamiltonians of the protonation states that makes the constant pH molecular dynamics simulations almost as fast as a normal MD simulation with GROMACS. In addition, we implemented a simpler scheme, called multisite representation, for modeling side chains with multiple titratable sites, such as imidazole rings. This scheme, which is based on constraining the

sum of the  $\lambda$ -coordinates, not only reduces the complexity associated with parameterizing the intra-molecular interactions between the sites, but is also easily extendable to other molecules with multiple titratable sites. With the combination of a more efficient interpolation scheme and multisite representation of titratable groups, we anticipate a rapid uptake of constant pH molecular dynamics simulations within the GROMACS user community.

## Introduction

Since their introduction more than four decades ago, molecular dynamics (MD) computer simulations have come of age.<sup>1</sup> Thanks to improvements in computer hardware, algorithmic developments, as well as increased accuracy of force fields, MD simulation has evolved into a predictive technique that can complement experiment by providing atomistic insights into the dynamics of complex systems.<sup>1,2</sup> While many experimental conditions can be modelled with good accuracy, the aqueous proton concentration, or pH, is typically accounted for indirectly by constraining the protonation states of titratable residues to their, presumed, most probable form at the start of the simulation. Because the electrostatic interactions depend critically on the protonation state of the residues, the pH affects the conformational ensemble. Conversely, because the conformation can influence the proton affinity of the residues, or  $pK_a$ , a direct correlation exists between pH and conformational dynamics, which cannot be captured if protonation state is kept fixed in the simulation.<sup>3</sup>

To overcome this limitation in classical MD simulations and include the effect of pH on the conformational sampling directly, several solutions have been proposed in the last decades.<sup>4,5</sup> These solutions can be roughly divided into a category that relies on discrete changes in protonation states,<sup>6-12</sup> and a second category, in which a protonation state can change continuously.<sup>13-25</sup> More recently, a third category that relies on the transfer of proton-like particles between titratable sites, including protein residues and solvent molecules, was proposed for the Martini force field.<sup>26</sup>

In the discrete constant pH approaches, the protonation state of a residue can change at regular intervals of the simulation according to a Metropolis Monte Carlo criterion.<sup>10,11,21,27,28</sup> To avoid a low acceptance rate due to unfavourable solvent configurations, the Monte Carlo step is performed based on free energies calculated using the approximation of either an implicit solvent representation,<sup>7,9</sup> or a short all-atom thermodynamic integration.<sup>8,29</sup>

Most continuous approaches for MD at constant pH are based on the  $\lambda$ -dynamics technique developed by Brooks and co-workers.<sup>30</sup> A one-dimensional  $\lambda$ -coordinate with fictitious mass  $m_\lambda$  is introduced for each titratable site, and the equations of motion for these additional degrees of freedom are integrated along with the Cartesian positions of the atoms.<sup>14</sup> The  $\lambda$ -coordinate defines the protonation state of the residue: at  $\lambda = 0$  the residue is protonated and interacts with the rest of the system as such, while at  $\lambda = 1$  it is deprotonated. The energy function that acts on the  $\lambda$ -coordinates depends on (i) the intrinsic proton affinity (reference  $pK_a$ ) of the titratable site in water, (ii) the interactions with the environment, which are mostly electrostatic,<sup>31</sup> and (iii) the pH of the solvent, which is set by the user. In addition, potentials are introduced to bias sampling towards the physical states at  $\lambda = 0$  and  $\lambda = 1$ . Protons are not transferred directly between the titratable residues and the solvent molecules, but rather exchanged with an external proton bath. Because the chemical potential of this bath is determined by the proton concentration (pH) of the aqueous solution, constant pH MD (CpHMD) simulations based on  $\lambda$ -dynamics are performed in a grand canonical ensemble for the proton degrees of freedom.

While  $\lambda$ -dynamics based constant pH approaches were originally developed for implicit solvent simulations,<sup>14</sup> they have since then been adapted for explicit solvent simulations as well.<sup>12,16–19,21,23</sup> The key computational challenge for explicit solvent implementations is the long-range electrostatic interaction, for which multiple solutions have been suggested, including a shifted cut-off scheme,<sup>19</sup> a hybrid scheme combining the particle mesh Ewald (PME) treatment for the Cartesian coordinates with the generalized Born model for the  $\lambda$  particles,<sup>17,32</sup> and a fully consistent PME treatment for both  $\lambda$  and Cartesian degrees of

freedom.<sup>16,23</sup>

In addition to an accurate modeling of the long-range electrostatic interactions, also sampling can pose a serious challenge to simulations at constant pH. While the choice for the PME method in the original implementation of  $\lambda$ -dynamics in the fork of GROMACS 3.3 release<sup>16</sup> was motivated by its accurate description of long-range electrostatics, the linear increase of the computational effort with the number of titratable sites limited the sampling efficiency, which meant that systems with many titratable sites could not be studied in practice.

To remove this bottleneck and enable constant pH MD with GROMACS at modest extra cost compared to a standard simulation, we switch to an alternative scheme for computing the long-range electrostatic interactions of the  $\lambda$ -particles under periodic boundary conditions. The alternative scheme is based on a linear interpolation of partial charges,<sup>14</sup> rather than the potential energy functions as in the original implementation of constant pH MD in a GROMACS fork.<sup>16</sup>

Although the previous implementation of constant pH in a GROMACS fork was documented and shared with the community as an open-source program, there has been some misunderstanding about how electrostatic interactions were computed for  $\lambda$ -particles.<sup>17,18,23</sup> To resolve this, we first explain in detail how the electrostatic interactions were calculated in the previous GROMACS implementation of constant pH MD. We next contrast this linear interpolation between the potential energy functions of the protonated and deprotonated states of a residue, with the interpolation between the partial charges of both states,<sup>14</sup> and show why the latter is computationally much more efficient. We then demonstrate the superior performance of the charge-interpolation scheme by running a series of constant pH MD simulations of amino acids and proteins. To emphasize that the new constant pH implementation in GROMACS is not linked to a specific force field or method for evaluating electrostatic interactions, we also show the results of constant pH MD simulations with the Martini coarse-grained force field.<sup>33</sup> Because of GROMACS' large user community, we expect

our work to increase popularity of constant pH MD simulations.

## Theory

Before discussing the differences between linear interpolating of the potential energy functions on the one hand,<sup>16</sup> and of partial charges on the other hand,<sup>14</sup> for computing the potential energy landscape of the titration coordinates, we briefly review the  $\lambda$ -dynamics approach<sup>30</sup> that forms the basis for the constant pH molecular dynamics algorithm in GRO-MACS.<sup>16</sup>

### $\lambda$ -dynamics based constant pH MD simulations

A titratable site  $i$  can exist in a protonated or deprotonated state. The protonation state affects the interactions between the site and the rest of the system. In constant pH MD simulations based on  $\lambda$ -dynamics,<sup>30</sup> an additional coordinate  $\lambda_i$  is introduced for each site  $i$ , and the potential energy function of the total system is continuously interpolated between the two protonation states along this coordinate *i.e.*,  $V(\lambda_i)$ .<sup>14</sup> A fictitious mass  $m_\lambda$  is assigned to each  $\lambda_i$ -coordinate, and the coordinates evolve along with the Cartesian degrees of freedom of all atoms in the system, based on Newton’s equations of motion. Thus, the total Hamiltonian of the system is

$$H(\mathbf{R}, \boldsymbol{\lambda}) = \sum_i^{N_{\text{sites}}} \frac{m_\lambda}{2} \dot{\lambda}_i^2 + \sum_j^{N_{\text{atoms}}} \frac{m_j}{2} \dot{\mathbf{r}}_j^2 + V(\mathbf{R}, \boldsymbol{\lambda}) \quad (1)$$

where  $\mathbf{R}$  is the vector of the Cartesian coordinates  $\mathbf{r}_j$  of all  $N_{\text{atoms}}$  atoms with mass  $m_j$ , and  $\boldsymbol{\lambda}$  is the vector of the  $\lambda_i$  coordinates of all  $N_{\text{sites}}$  titratable sites.

### $\lambda$ -dependent potential energy function

In addition to the interpolation between the potentials of the protonated  $V_A(\mathbf{R})$  and deprotonated states  $V_B(\mathbf{R})$ , three more  $\lambda$ -dependent terms are included in the potential energy

function of the total system  $V(\mathbf{R}, \boldsymbol{\lambda})$ , as illustrated in Figure 1: (i) a correction potential  $V_i^{\text{MM}}(\lambda_i)$  to compensate for missing quantum mechanical contributions to proton affinities (Figure 1B); (ii) a biasing potential  $V_i^{\text{bias}}(\lambda_i)$  that enhances sampling of the physical end states at  $\lambda_i = 0$  and  $\lambda_i = 1$  (Figure 1C); and (iii) a pH-dependent term  $V^{\text{pH}}(\lambda_i)$  to model the chemical potential of protons in water (Figure 1D).

The purpose of adding the correction term  $V_i^{\text{MM}}(\lambda_i)$  (Figure 1B) is to make the interpolated potential function flat if the titratable site  $i$  is in its reference state, for which the proton affinity is known experimentally, at  $\text{pH} = \text{p}K_{\text{a},i}$ . This potential is determined by evaluating the deprotonation free energy of the single residue in water (reference state) at the force field level by thermodynamic integration along the  $\lambda$ -coordinate (Figure 1A):

$$V_i^{\text{MM}}(\lambda_i) = -\Delta G_i^{\text{MM}}(\lambda_i) \quad (2)$$

To prevent sampling of the non-physical states between  $\lambda_i = 0$  and  $\lambda_i = 1$  on this flat potential energy surface while still enabling sufficient transitions between the physical end-states to sample both protonation states with the correct thermodynamic weight, we introduce the biasing potential  $V_i^{\text{bias}}(\lambda_i)$  suggested by Donnini *et al.*<sup>34</sup> (Figure 1C).

The pH dependent term  $V^{\text{pH}}(\lambda)$  (Figure 1D) is a correction to include the effect of the solution pH on the free energy difference between the protonated and deprotonated states, such that this difference is

$$V^{\text{pH}}(\lambda_i = 1) - V^{\text{pH}}(\lambda_i = 0) = RT \ln(10) [\text{p}K_{\text{a},i} - \text{pH}] \quad (3)$$

where we use the experimentally determined  $\text{p}K_{\text{a},i}$  values of residue  $i$  in its reference state. Although various forms for this potential have been suggested,<sup>22,34,35</sup> we propose a smooth

step-function-based potential:

$$V^{\text{pH}}(\lambda_i) = RT \ln(10) [\text{p}K_{\text{a},i} - \text{pH}] \frac{1}{1 + \exp(-2k_1(\lambda_i - 1 + x_0))}, \text{ if } \text{pH} > \text{p}K_{\text{a}}$$

$$V^{\text{pH}}(\lambda_i) = RT \ln(10) [\text{p}K_{\text{a},i} - \text{pH}] \frac{1}{1 + \exp(-2k_1(\lambda_i - x_0))}, \text{ if } \text{pH} \leq \text{p}K_{\text{a}}, \quad (4)$$

where  $k_1$  and  $x_0$  define the steepness and kink position of the step function. In this form, illustrated in Figure 1D, the pH dependent potential also aids in preventing the sampling of non-physical states, *i. e.*,  $0.1 < \lambda < 0.9$ .

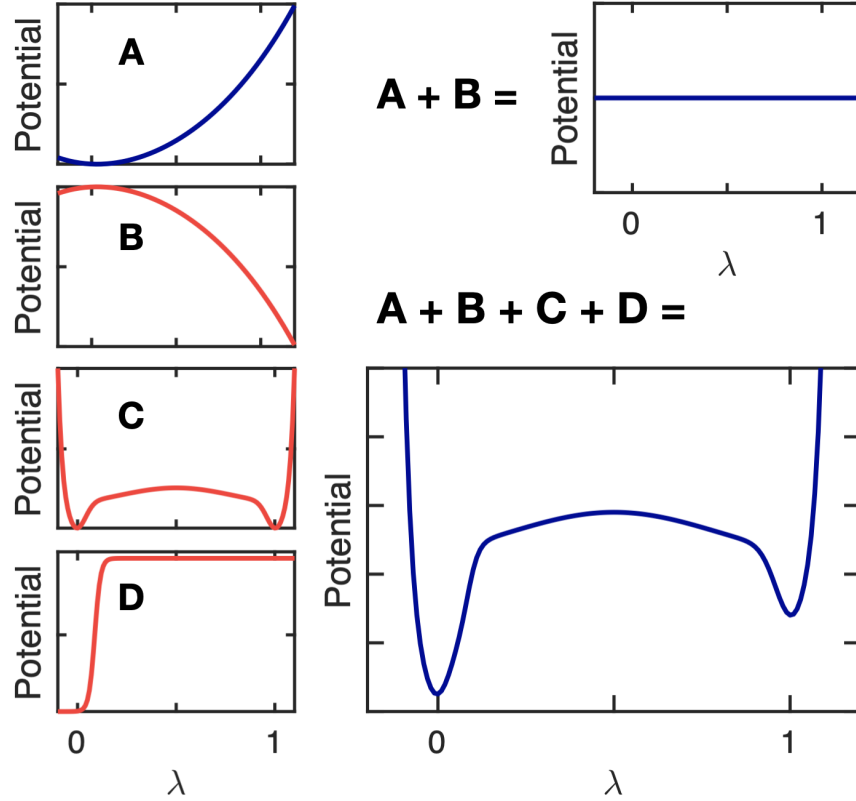


Figure 1: Illustration of the additional  $\lambda$ -dependent potential energy terms (B-D). Panel A shows the protonation free energy of a titratable residue in its reference state obtained at the force field level,  $\Delta G_i^{\text{MM}}(\lambda)$ . To compensate for the shortcomings of the force field and obtain a zero free energy difference between the two protonation states (A+B), we add the correction potential,  $V_i^{\text{MM}}(\lambda)$ , shown in panel B. A biasing potential,  $V^{\text{bias}}(\lambda)$ ,<sup>34</sup> is introduced to avoid sampling of non-physical states (panel C). To model the proton chemical potential (pH), we add a pH dependent term,  $V^{\text{pH}}(\lambda_i)$  (panel D). For a titratable residue at  $\text{pH} \neq \text{p}K_{\text{a}}$ , the total  $\lambda$ -dependent potential, including the interpolated force field functions and the three additional terms, is illustrated in panel (A+B+C+D).

## Linear interpolation of potential energy functions

In the previous implementation of constant pH MD in GROMACS’ fork, the smooth interpolation of the force field potential energy function between the protonated and deprotonated states was achieved by linearly interpolating the force field potentials of these states.<sup>16</sup> Thus, for a *single* titratable site, the  $\lambda$ -dependent potential is given by

$$V(\mathbf{R}, \lambda) = (1 - \lambda)V_A(\mathbf{R}) + \lambda V_B(\mathbf{R}) + V^{\text{MM}}(\lambda) + V^{\text{bias}}(\lambda) + V^{\text{pH}}(\lambda) \quad (5)$$

with  $V^{\text{MM}}(\mathbf{R}, \lambda)$ ,  $V^{\text{bias}}(\lambda)$  and  $V^{\text{pH}}(\lambda)$  the correction, biasing, and pH dependent potentials, respectively, that were briefly discussed above, and with a short-hand notations for

$$V_A(\mathbf{R}) = V(\mathbf{R}, \lambda = 0)$$

$$V_B(\mathbf{R}) = V(\mathbf{R}, \lambda = 1)$$

The gradient required for updating  $\lambda$  according to Newton’s equations of motion is

$$\frac{\partial V(\mathbf{R}, \lambda)}{\partial \lambda} = V_B(\mathbf{R}) - V_A(\mathbf{R}) + \frac{\partial}{\partial \lambda} V^{\text{MM}}(\mathbf{R}, \lambda) + \frac{\partial}{\partial \lambda} V^{\text{bias}}(\lambda) + \frac{\partial}{\partial \lambda} V^{\text{pH}}(\lambda) \quad (6)$$

Thus, the evaluation of the force on the  $\lambda$ -particle requires that the potential energy, including the long-range electrostatic interactions, is computed twice: once for  $\lambda = 0$  (*i.e.*,  $V_A$ ) and once more for  $\lambda = 1$  (*i.e.*,  $V_B$ ). If the Particle-Mesh-Ewald (PME) method is used to compute those long-range electrostatic interactions,<sup>36,37</sup> separate PME grid builds are needed, because the charge distributions are not identical in states  $A$  and  $B$ .

For systems with many titratable sites, multiple  $\lambda$ -groups are introduced. Because the analytical expressions for the correction, biasing and pH dependent terms in equation 5 are additive, we no longer consider them explicitly in what follows and focus exclusively on the interpolation of the force field potential energies between the multiple protonation states of the system. For  $N$   $\lambda$ -coordinates, there are  $2^N$  such states and the interpolation generalizes



to:<sup>13</sup>

$$V(\mathbf{R}, \boldsymbol{\lambda}) = \sum_{\mathbf{l}}^{2^N} V(\mathbf{R}, \mathbf{l}) \prod_i^N \lambda_i^{(l_i)} (1 - \lambda_i)^{1-l_i} \quad (7)$$

Here, we represent the  $N$   $\lambda_i$ -coordinates as an  $N$ -dimensional vector  $\boldsymbol{\lambda}$ . The  $2^N$  possible protonation states of the system are represented by the  $N$ -dimensional vector  $\mathbf{l}$  with elements  $l_i$  equal to 0 or 1 that indicate whether a site  $i$  is protonated ( $\lambda_i = 0$ ) or deprotonated ( $\lambda_i = 1$ ). The sum runs over all  $2^N$  possible combinations of  $l_i = 0$  and  $l_i = 1$ . The gradient required for updating  $\lambda_i$  is obtained by deriving the interpolated potential,  $V(\mathbf{R}, \boldsymbol{\lambda})$ , with respect to  $\lambda_i$ :

$$\begin{aligned} \frac{\partial}{\partial \lambda_i} V(\mathbf{R}, \boldsymbol{\lambda}) &= \frac{\partial}{\partial \lambda_i} \left[ \sum_{\mathbf{l}}^{2^N} V(\mathbf{R}, \mathbf{l}) \prod_k^N \lambda_k^{(l_k)} (1 - \lambda_k)^{1-l_k} \right] \\ &= \frac{\partial}{\partial \lambda_i} \left[ \sum_{\mathbf{l}', \mathbf{l}_i=0}^{2^{N-1}} V(\mathbf{R}, \mathbf{l}) \prod_k^N \lambda_k^{(l_k)} (1 - \lambda_k)^{1-l_k} (1 - \lambda_i) \right. \\ &\quad \left. + \sum_{\mathbf{l}', l_i=1}^{2^{N-1}} V(\mathbf{R}, \mathbf{l}) \prod_k^N \lambda_k^{(l_k)} (1 - \lambda_k)^{1-l_k} \lambda_i \right] \\ &= \sum_{\mathbf{l}', l_i=1}^{2^{N-1}} V(\mathbf{R}, \mathbf{l}) \prod_k^N \lambda_k^{(l_k)} (1 - \lambda_k)^{1-l_k} \\ &\quad - \sum_{\mathbf{l}', l_i=0}^{2^{N-1}} V(\mathbf{R}, \mathbf{l}') \prod_k^N \lambda_k^{(l_k)} (1 - \lambda_k)^{1-l_k} \\ &= V(\mathbf{R}, \boldsymbol{\lambda}', \lambda_i = 1) - V(\mathbf{R}, \boldsymbol{\lambda}', \lambda_i = 0) \end{aligned} \quad (8)$$

where the  $'$  indicates that  $\lambda_i$  is omitted from vector  $\boldsymbol{\lambda}$ . Note that, as we focus only on the interpolated potentials, the biasing, correction and pH-dependent terms are left out.

In general, the number of terms in the potential (Equation 7) increases exponentially with the number of titratable sites. However, for pair-wise interactions involving titratable sites whose non-bonded force field parameters do not depend on the protonation state of the other sites (chemically uncoupled sites), the number of terms required to evaluate the interpolated potential scales linearly. For systems with such "chemically", or "topologically"

uncoupled sites, the interpolated potential contains four types of interactions

$$V(\mathbf{R}, \boldsymbol{\lambda}) = V^{\text{rest-rest}}(\mathbf{R}) + V^{\boldsymbol{\lambda}\text{-rest}}(\mathbf{R}, \boldsymbol{\lambda}) + V^{\boldsymbol{\lambda}\text{-}\boldsymbol{\lambda}}(\mathbf{R}, \boldsymbol{\lambda}) + V^{\boldsymbol{\lambda}}(\mathbf{R}, \boldsymbol{\lambda}) \quad (9)$$

For pair-wise electrostatic interactions, the terms on the right-hand side are defined as:

1. Interactions between atoms that are not part of any  $\lambda$ -group, and hence independent of the  $\lambda_i$ 's:

$$V^{\text{rest-rest}}(\mathbf{R}) = \frac{1}{2} \sum_i^{n_{\text{rest}}} \sum_j^{n_{\text{rest}}} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} \quad (10)$$

where the sums run over all  $n_{\text{rest}}$  atoms that are not part of a  $\lambda$  group.

2. Interpolated interactions between atoms of each  $\lambda$ -group with atoms that are not part of any  $\lambda$ -group:

$$V^{\boldsymbol{\lambda}\text{-rest}}(\mathbf{R}, \boldsymbol{\lambda}) = \sum_k^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_{\text{rest}}} (1 - \lambda_k) \frac{q_i^A q_j}{4\pi\epsilon_0 r_{ij}} + \lambda_k \frac{q_i^B q_j}{4\pi\epsilon_0 r_{ij}} \quad (11)$$

where the first sum runs over all titratable sites, the second one runs over all  $n_k$  atoms of the  $k$ -th  $\lambda$ -group and final sum runs over all atoms that are not part of any  $\lambda$  group.

3. Interpolated interactions between atoms belonging to two different  $\lambda$ -groups:

$$\begin{aligned} V^{\boldsymbol{\lambda}\text{-}\boldsymbol{\lambda}}(\mathbf{R}, \boldsymbol{\lambda}) &= \sum_k^{N_{\text{sites}}} \left[ (1 - \lambda_k) \sum_{m, m \neq k}^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \left[ (1 - \lambda_m) \frac{q_i^A q_j^A}{4\pi\epsilon_0 r_{ij}} + \lambda_m \frac{q_i^A q_j^B}{4\pi\epsilon_0 r_{ij}} \right] \right. \\ &\quad \left. + \lambda_k \sum_{m, m \neq k}^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \left[ (1 - \lambda_m) \frac{q_i^B q_j^A}{4\pi\epsilon_0 r_{ij}} + \lambda_m \frac{q_i^B q_j^B}{4\pi\epsilon_0 r_{ij}} \right] \right] \\ &= \sum_k^{N_{\text{sites}}} \sum_{m, m \neq k}^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \frac{[(1 - \lambda_k) q_i^A + \lambda_k q_i^B][(1 - \lambda_m) q_j^A + \lambda_m q_j^B]}{4\pi\epsilon_0 r_{ij}} \end{aligned} \quad (12)$$

4. Interpolated interactions within each of the  $\lambda$ -groups:

$$V^\lambda(\mathbf{R}, \lambda) = \frac{1}{2} \sum_k^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_k} (1 - \lambda_k) \frac{q_i^A q_j^A}{4\pi\epsilon_0 r_{ij}} + \lambda_k \frac{q_i^B q_j^B}{4\pi\epsilon_0 r_{ij}} \quad (13)$$

From equation 8 the gradient with respect to  $\lambda_k$  is:

$$\begin{aligned} \frac{\partial}{\partial \lambda_k} V_{\text{Coul}}(\mathbf{R}, \lambda) &= V_{\text{Coul}}(\mathbf{R}, \lambda', \lambda_k = 1) - V_{\text{Coul}}(\mathbf{R}, \lambda', \lambda_k = 0) \\ &= \sum_i^{n_k} \sum_j^{n_{\text{rest}}} \frac{q_i^B q_j}{4\pi\epsilon_0 r_{ij}} - \frac{q_i^A q_j}{4\pi\epsilon_0 r_{ij}} \\ &\quad + \sum_m^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \frac{q_i^B [(1-\lambda_m)q_j^A + \lambda_m q_j^B]}{4\pi\epsilon_0 r_{ij}} - \frac{q_i^A [(1-\lambda_m)q_j^A + \lambda_m q_j^B]}{4\pi\epsilon_0 r_{ij}} \\ &\quad + \frac{1}{2} \sum_i^{n_k} \sum_j^{n_k} \frac{q_i^B q_j^B}{4\pi\epsilon_0 r_{ij}} - \frac{q_i^A q_j^A}{4\pi\epsilon_0 r_{ij}} \end{aligned} \quad (14)$$

Thus, the evaluation of the Coulomb contribution to the gradient for each  $\lambda_k$ -group requires two electrostatic computations, with the interpolated partial charges of the other  $\lambda_m$  sites (*i.e.*,  $q_j(\lambda_m) = (1 - \lambda_m)q_j^A + \lambda_m q_j^B$ ):

$$\begin{aligned} \frac{\partial}{\partial \lambda_k} V_{\text{Coul}}(\mathbf{R}, \lambda) &= \sum_i^{n_k} q_i^B \times \left[ \sum_a^{n_{\text{rest}}} \frac{q_a}{4\pi\epsilon_0 r_{ia}} \right. \\ &\quad \left. + \sum_m^{N_{\text{sites}}} \sum_j^{n_m} \frac{(1-\lambda_m)q_j^A + \lambda_m q_j^B}{4\pi\epsilon_0 r_{ij}} + \frac{1}{2} \sum_j^{n_k} \frac{q_j^B}{4\pi\epsilon_0 r_{ij}} \right] \\ &\quad - \sum_i^{n_k} q_i^A \times \left[ \sum_a^{n_{\text{rest}}} \frac{q_a}{4\pi\epsilon_0 r_{ia}} \right. \\ &\quad \left. + \sum_m^{N_{\text{sites}}} \sum_j^{n_m} \frac{(1-\lambda_m)q_j^A + \lambda_m q_j^B}{4\pi\epsilon_0 r_{ij}} + \frac{1}{2} \sum_j^{n_k} \frac{q_j^A}{4\pi\epsilon_0 r_{ij}} \right] \\ &= \sum_i^{n_k} q_i^B \Phi_k^B(\mathbf{R}_i, \lambda') - \sum_i^{n_k} q_i^A \Phi_k^A(\mathbf{R}_i, \lambda') \end{aligned} \quad (15)$$

Here, we introduced the electrostatic potential  $\Phi_k^A(\mathbf{R}_i, \lambda')$  of the system with partial charges

of  $\lambda$ -group  $k$  in the protonated state ( $q_i^A$ ) and interpolated charges for all other  $\lambda$ -groups. As before,  $\boldsymbol{\lambda}'$  is the vector with all  $\lambda_m$ 's except  $\lambda_k$ . Likewise, electrostatic potential  $\Phi_k^B(\mathbf{R}_i, \boldsymbol{\lambda}')$  is evaluated with the partial charges of  $\lambda$ -group  $k$  in the deprotonated state ( $q_i^B$ ) and the same interpolated charges for all other  $\lambda$ -groups. Thus,  $2N_{\text{sites}}$  computations are needed to evaluate the gradients for all titratable sites. The same arguments apply to pair-wise Lennard-Jones interactions, but because the contribution of Lennard-Jones interaction to  $pK_a$  shift is minor, we neglected them in this work (see Supporting Information).

### Linear interpolation of partial charges

While the linear scaling of the gradients for the pair-wise potentials in equation 15 in principle is a great improvement over the formal exponential scaling in equation 8, the requirement of performing  $2N_{\text{sites}}$  calculations per MD step still poses a computational bottleneck, in particular for larger systems. To overcome this bottleneck for electrostatic interactions, we follow the suggestion by Brooks and co-workers to interpolate charges rather than interaction functions.<sup>14</sup> When interpolating the partial charges between the protonation states of  $N_{\text{sites}}$  chemically uncoupled titratable sites, the  $\lambda$ -dependent Coulomb energy becomes:

$$\begin{aligned}
V_{\text{coul}}(\mathbf{R}, \boldsymbol{\lambda}) &= V^{\text{rest-rest}}(\mathbf{R}) + V^{\lambda\text{-rest}}(\mathbf{R}, \boldsymbol{\lambda}) + V^{\lambda\text{-}\lambda}(\mathbf{R}, \boldsymbol{\lambda}) + V^{\lambda}(\mathbf{R}, \boldsymbol{\lambda}) \\
&= \frac{1}{2} \sum_i^{n_{\text{rest}}} \sum_j^{n_{\text{rest}}} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} \\
&\quad + \sum_k^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_{\text{rest}}} \frac{((1-\lambda_k)q_i^A + \lambda_k q_i^B) q_j}{4\pi\epsilon_0 r_{ij}} \\
&\quad + \sum_k^{N_{\text{sites}}} \sum_{m; m \neq k}^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \frac{[(1-\lambda_k)q_i^A + \lambda_k q_i^B][(1-\lambda_m)q_j^A + \lambda_m q_j^B]}{4\pi\epsilon_0 r_{ij}} \\
&\quad + \frac{1}{2} \sum_k^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_k} \frac{[(1-\lambda_k)q_i^A + \lambda_k q_i^B][(1-\lambda_k)q_j^A + \lambda_k q_j^B]}{4\pi\epsilon_0 r_{ij}}
\end{aligned} \tag{16}$$

The gradient of the potential energy with respect to  $\lambda_k$  is

$$\begin{aligned}
\frac{\partial V_{\text{coul}}(\mathbf{R}, \boldsymbol{\lambda})}{\partial \lambda_k} &= \sum_i^{n_k} \sum_j^{n_{\text{rest}}} \frac{(q_i^B - q_i^A) q_j}{4\pi\epsilon_0 r_{ij}} \\
&\quad + \sum_{m=1; m \neq k}^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \frac{(q_i^B - q_i^A) [(1 - \lambda_m) q_j^A + \lambda_m q_j^B]}{4\pi\epsilon_0 r_{ij}} \\
&\quad + \sum_i^{n_k} \sum_j^{n_k} \frac{(q_i^B - q_i^A) [(1 - \lambda_k) q_j^A + \lambda_k q_j^B]}{4\pi\epsilon_0 r_{ij}} \\
&= \sum_i^{n_k} \sum_j^{n_{\text{rest}}} \frac{(q_i^B - q_i^A) q_j}{4\pi\epsilon_0 r_{ij}} \\
&\quad + \sum_{m=1}^{N_{\text{sites}}} \sum_i^{n_k} \sum_j^{n_m} \frac{(q_i^B - q_i^A) [(1 - \lambda_m) q_j^A + \lambda_m q_j^B]}{4\pi\epsilon_0 r_{ij}} \\
&= \sum_i^{n_k} \Phi(\mathbf{R}_i, \boldsymbol{\lambda}) \Delta q_i
\end{aligned} \tag{17}$$

where  $\Phi(\mathbf{R}_i, \boldsymbol{\lambda})$  is the electrostatic potential at the position of atom  $i$  due to the charge distribution of all other atoms in the system, including the atoms of all titratable sites, for which the partial charges are interpolated:

$$\Phi(\mathbf{R}_i, \boldsymbol{\lambda}) = \sum_j^{n_{\text{rest}}} \frac{q_j}{4\pi\epsilon_0 r_{ij}} + \sum_m^{N_{\text{sites}}} \sum_j^{n_m} \frac{(1 - \lambda_m) q_j^A + \lambda_m q_j^B}{4\pi\epsilon_0 r_{ij}}$$

and  $\Delta q_i$  is the difference between the atomic charges of titratable residue  $i$  in the protonated (A) and deprotonated (B) states:

$$\Delta q_i = q_i^B - q_i^A$$

In contrast to when potential energy functions are interpolated, the same electrostatic potential is used to evaluate the electrostatic forces on both the atoms and the  $\lambda$ -particles. Therefore, a *single* electrostatic calculation per time step suffices. If the electrostatic interactions are modeled with the smooth Particle Mesh Ewald method,<sup>36,37</sup> the short-range real-space interactions and long-range reciprocal-space interactions are computed separately. For the

pair-wise short-range interactions in real space an additional calculation for each interacting pair and a subsequent accumulation of the potential at each atom is needed. Whereas this calculation comes at no extra computational cost if the standard pair interaction kernels are used, the accumulation leads to a measurable computational overhead, as we will show later. For the mesh part of the PME calculation, a gathering of potentials from the grid is required for charges in  $\lambda$ -groups only, but this also comes at a negligible computational overhead. Because the extra effort required to compute the gradients on the  $\lambda$  particles is rather small, a constant pH MD implementation based on charge interpolation is computationally not much more expensive than a normal MD simulation, which is a major improvement with respect to the previous CpHMD implementation in GROMACS.<sup>16</sup>

## Multisite representation of *chemically coupled* titratable sites

If titratable sites are "chemically" or "topologically" coupled, the force field parameters of one site depend on the value of the  $\lambda$  coordinate of the other site, and *vice versa*. For example, histidine can exist in three protonation states, as shown in Figure 2. In most force fields, the partial charges of all atoms in the His side chain, including the two sites, depend on the protonation state. Hence, if the  $N_\delta$  site changes protonation, also the electrostatic interactions of the  $N_\epsilon$  site are affected.

To model the chemically coupled sites in the histidine side chain, Khandogin and Brooks introduced two  $\lambda$ -coordinates:<sup>15</sup> one that interpolates between the double and single protonated forms and a second coordinate switching between protonation at the  $N_\delta$  and the  $N_\epsilon$  atoms. Donnini *et al.* introduced separate  $\lambda$ -coordinates for  $N_\delta$  and  $N_\epsilon$ .<sup>16</sup> In both solutions, the coupling between the coordinates is achieved with a two-dimensional correction potential.

Because extending the dimensionality beyond two coordinates is difficult from both the implementation and parameterization perspective, Brooks and co-workers introduced a multisite representation,<sup>38,39</sup> where a separate  $\lambda_{i,k}$ -coordinate is assigned to each physical state

$k$  of a titratable group  $i$ . For a residue with multiple "chemically"-coupled titratable sites each  $\lambda_{i,k}$ -coordinate has the same state at  $\lambda_{i,k} = 0$ , while at  $\lambda_{i,k} = 1$  the group is in one of the  $n_i$  possible protonation states (*i.e.*, state  $k$ ) of residue  $i$ . The state at  $\lambda_{i,k} = 0$  (for all coordinates  $k$  of residue  $i$ ) is the same for all  $\lambda$ -coordinates but does not correspond to a physical protonation state of the residue, and neither do states for which the sum of the  $\lambda_{i,k}$ -s is not equal to 1 (Figure 2). To restrict sampling to the (hyper-)plane connecting the physical states, the sum of  $\lambda_{i,k}$ -s is constrained ( $\sum_k \lambda_{i,k} = 1$ ). Since the  $\lambda$ -dynamics implementation in a fork of GROMACS relies on linear  $\lambda$ -coordinates, rather than the auxiliary circular coordinates that would fulfill the constraints by construction,<sup>38</sup> we apply a constraint on the sum of  $\lambda$ -s. To efficiently apply this constraint we use an analytical expression to solve a generalized version of charge constraint introduced by Donnini *et al.*<sup>34</sup> (see Appendix). While an atom can be part of multiple  $\lambda_{i,k}$  coordinates in residue  $i$ , each affecting its charge, we show in SI that the expression for the contribution of this atom to the total Coulomb energy is identical to that of an uncoupled site (equation 17).

In the multisite representation, each  $\lambda$ -coordinate is independent of the others and thus evolves on a one dimensional potential (equation 4), similar to that of "chemically" uncoupled sites. However, in contrast to the uncoupled sites, the correction potential  $V^{\text{MM}}$  is multi-dimensional as its value depends on all  $\lambda_{i,k}$  coordinates representing each of the possible protonation states of residue  $i$ . These potentials are obtained through a least-square fit of a multi-dimensional polynomial to the ensemble-averaged gradients of the potentials with respect to  $\lambda_{i,k}$  evaluated on the  $(n_i - 1)$ -dimensional grid of the  $n_i$  coupled  $\lambda$ -s, *i. e.*:  $\langle \partial V / \partial \lambda_{i,k} \rangle_{\lambda_1 \dots \lambda_{n_i}}$ . The fitting procedure is explained in detail in the SI.

The multisite representation can be applied to residues with any number of titratable sites, including residues with only a single titratable site. In the latter case, two  $\lambda$ -coordinates, corresponding to the protonated state ( $\lambda_{i,1} = 1, \lambda_{i,2} = 0$ ) and deprotonated state ( $\lambda_{i,1} = 0, \lambda_{i,2} = 1$ ) are introduced with a constraint on their sum ( $\lambda_{i,1} + \lambda_{i,2} = 1$ ).

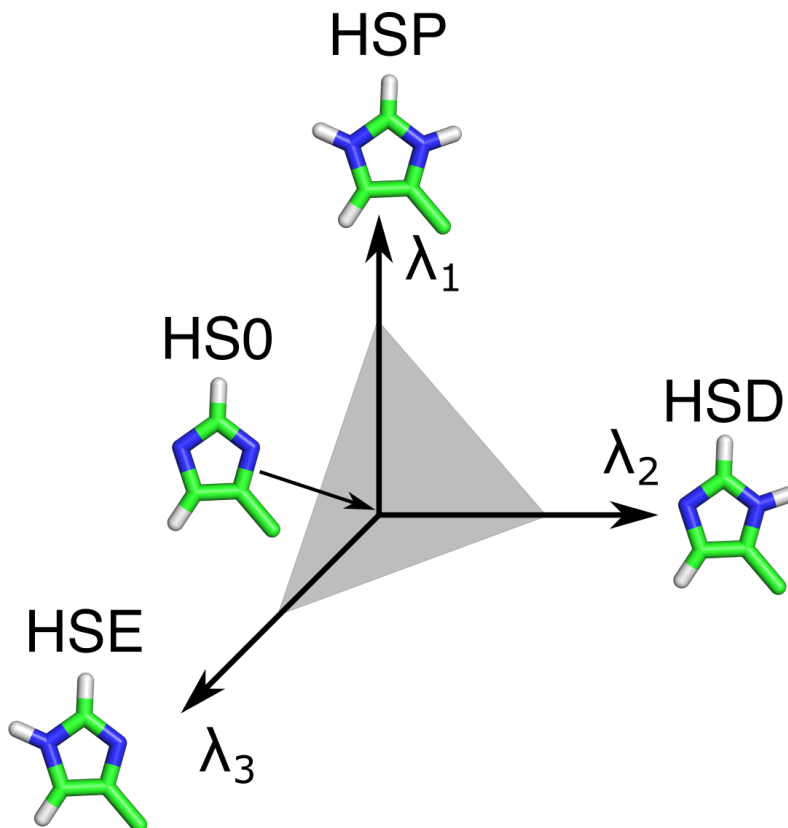


Figure 2: Multisite representation illustrated for a histidine side chain. Each possible protonation state is represented by its own  $\lambda$ -coordinate. HSP refers to doubly protonated histidine, HSD and HSE to histidine singly protonated at the  $N_\delta$  or  $N_\epsilon$  respectively. HS0 is a common, non-physical state of the residue. To restrict the sampling to the plane connecting the physical states, a constraint  $\lambda_1 + \lambda_2 + \lambda_3 = 1$  is applied (gray plane). A biasing potential is also applied to enhance sampling at the end states, where one of the  $\lambda$ -coordinates is close to one, while the other coordinates are close to zero.

## Methods

We have implemented the algorithms for CpHMD with charge interpolation in a fork of GROMACS software package (2021 release).<sup>40</sup> The code and manuals are available for free at <https://gitlab.com/gromacs-constantph/constantph>. Here we verify the validity of our implementation for reproducing  $pK_a$  values of peptides and proteins. To demonstrate that the linear interpolation of charges (equation 17) scales better with the number of titratable sites in the system than the linear interpolation of interaction functions (equation 15), we compared the scaling between our new implementation, which is based on linear charge



interpolation on the one hand, and a previous implementation in a fork of the GROMACS 3.3 release, which is based on linear interpolation of the force field potentials on the other hand.<sup>16</sup> To estimate the additional computational effort required for performing CpHMD with the new implementation, we also compared the performance of a CpHMD simulation to that of a normal MD simulations on both CPUs and GPUs.

## Simulated systems

To test the implementation, we performed constant pH MD simulations of the following systems:

1. Glutamic acid (Glu)
2. Aspartic acid (Asp)
3. Histidine (His)
4. Cardiotoxin V (PDB ID: 1CVO<sup>41</sup>)
5. Hen egg white lysozyme (HEWL, PDB ID: 2LZT<sup>42</sup>)
6. The GLIC pentameric Ligand-Gated Ion Channel (PDB ID: 4HFI<sup>43</sup>)
7. Turkey ovomucoid inhibitor (PDB ID: 2GKR<sup>44</sup>)

In systems 1-6 the interactions were modelled with the CHARMM36<sup>45</sup> all-atom (AA) force field, with some modifications in the torsion parameters to accelerate the convergence. These modifications are presented and validated in an accompanying paper.<sup>46</sup> Systems 1-5 were also simulated with the Martini 2.0 coarse grained (CG) force field.<sup>33</sup> The martinize.py script was used to automatically generate the CG representation of these systems.<sup>47</sup> System 7 was simulated to compare the efficiency of interpolating charges and potentials. The interactions in this system were modeled with the OPLS force field,<sup>48</sup> because the GROMACS

3.3 release, on which the linear interpolation of potentials implementation was based, does not support the CMAP correction that is needed for the CHARMM36 force field.<sup>49</sup>

The amino acids Glu, Asp and His were modelled as tripeptides Ala-X-Ala with acetylated N-terminus (ACE) and N-methylamidated C-terminus (CT<sub>3</sub>). The proteins were simulated with charged termini. The tripeptides were placed in a periodic rectangular box of dimensions  $5 \times 5 \times 5 \text{ nm}^3$  with approximately 4000 CHARMM TIP3P<sup>50,51</sup> water molecules for the AA simulations and 950 polarizable water particles for the CG simulations.<sup>52</sup> The water soluble protein cardiotoxin V was placed in a periodic rectangular box of  $7.9 \times 7.9 \times 7.9 \text{ nm}^3$  and filled with 16500 CHARMM TIP3P water molecules for the AA simulations. For the CG simulations, the protein was placed inside a periodic rectangular box of  $5.7 \times 5.7 \times 5.7 \text{ nm}^3$  and filled with 1800 polarizable water particles. The larger water soluble protein HEWL was placed in a periodic rectangular box of  $8.9 \times 8.9 \times 8.9 \text{ nm}^3$  and filled with 23000 CHARMM TIP3P water molecules for the AA simulations and 5400 polarizable water particles for the CG simulations. Na<sup>+</sup> and Cl<sup>-</sup> ions were added to all systems at 0.15 M concentration to neutralize the protein systems. The turkey ovomucoid inhibitor protein was placed in a box of  $4.9 \times 4.9 \times 4.9 \text{ nm}^3$  with 3086 SPC<sup>53</sup> water molecules. The GLIC protein was embedded into a bilayer membrane containing 498 phosphatidylcholine (POPC) lipids, placed in a box of  $14.0 \times 14.0 \times 15.9 \text{ nm}^3$ , and filled with 66494 CHARMM TIP3P waters, 58 Na<sup>+</sup>, 123 Cl<sup>-</sup> ions. The system contained 292135 atoms in total. The simulation of this system was performed with the GROMACS 2021.4 release as reference. The GLIC benchmarks were run with default settings on an Intel i9-7920X 12-core CPU and an Nvidia RTX 2080 Ti GPU. All input configurations are provided as Supporting Information.

In the AA simulations, Coulomb interactions were modeled with the smooth PME method with a real-space cut-off of 1.2 nm and a grid spacing of 0.14 nm,<sup>36,37</sup> while Lennard-Jones interactions were smoothly switched to zero in range from 1.0 to 1.2 nm. In the CG simulations, Coulomb interactions were modeled by a Reaction Field potential with a 1.1 nm cut-off,  $\epsilon_r = 2.5$ , and  $\epsilon_{\text{RF}} = \infty$ , while Lennard-Jones interactions were truncated at 1.1 nm.<sup>54</sup>

To keep the temperature constant at 300 K, we used the v-rescale thermostat<sup>55</sup> with time constants of 0.5 and 1.0 ps<sup>-1</sup> for AA and CG simulations, respectively. The pressure was kept constant at 1 bar with the Parrinello-Rahman barostat<sup>56</sup> with relaxation times of 2.0 and 12.0 ps for AA and CG simulations, respectively. A leapfrog integrator was used with an integration steps of 2 and 20 fs for AA and CG simulations, respectively. In the AA simulations, the LINCS algorithm<sup>57</sup> was used to constrain bond lengths of the solutes, while the SETTLE<sup>58</sup> algorithm was used to constrain internal degrees of freedom of the water molecules. Prior to the constant pH MD simulations, the potential energy of each system was minimized using the steepest descent method, followed by 1 ns of equilibration.

## Constant pH MD simulation setups

In the atomistic simulations the multisite representation was used to model the protonation states of titratable residues. Two  $\lambda$ -coordinates were introduced to model the two forms of the carboxylic acid side chain in Asp and Glu, while three coordinates were used to describe the three protonation states of the imidazole sidechain in His. In the CG simulations, the single-site representation was used, in which the A and B states represent the protonated and deprotonated states of the titratable beads. Because, in contrast to AA force fields, there is no distinction between the two neutral forms of the His sidechain in the Martini force field, the single-site description for HIS suffices in the CG simulations. In both atomistic and coarse-grained simulations, the transformations between the different protonation states were achieved by changing the charges of the ionizable groups. The Lennard-Jones and bonded terms (bonds, angles, and torsions) were kept in the protonated and deprotonated state in AA and CG simulations, respectively (see Supplementary Information, Fig. S3). We show in SI that the contribution of these terms is sufficiently small to be neglected without significant error. Note, that these terms can be made  $\lambda$ -dependent as well, but this is beyond the scope of the current work since the efforts to implement this are high.

The mass of the  $\lambda$ -particles was set to 5 atomic units, and their temperature was main-

tained at 300 K by using a separate v-rescale thermostat for the  $\lambda$  coordinates with a time constant of 2.0 ps<sup>-1</sup>. For all  $\lambda$ -coordinates the biasing potential  $V_i^{\text{bias}}(\lambda_i)$  was defined by equation S1 in the SI. The barrier height of the double well potential was set to 5.0 and 7.5 kJ/mol for AA and CG simulations, respectively. The parameters for the double well potential and the pH dependent potential (Equation 4) are provided in Table S1 in the SI.

For the tripeptides, we calculated five independent CpHMD trajectories of 20 ns each at 13 pH values, ranging from 1.0 to 7.0 for the peptides with Glu and Asp, and from 4.0 to 10.0 for the peptides with His. For the cardiotoxin V protein (three Asp and one His titratable residues), we performed five independent CpHMD simulations of 50 ns at 15 pH values between 1.0 to 8.0. For the HEWL protein (seven Asp, two Glu and one His titratable residues), we performed five independent CpHMD simulations of 75 ns at 21 pH values between -1.0 to 9.0. The values of the  $\lambda$ -coordinates were written to file with a frequency of 1 ps<sup>-1</sup>.

### Reference states and force field correction potentials

The constant pH simulations of the aforementioned systems require reference states for Asp, Glu and His, in which the proton affinity ( $\text{p}K_{\text{a}}$ ) is known from experiment. The measured and calculated (force field) deprotonation free energies of these reference states were used to include the effect of the pH bath, as well as the effects of the breaking and forming of chemical bonds in the simulation, *i.e.*,  $V^{\text{MM}}$  in Equation 2. The measured reference  $\text{p}K_{\text{a}}$  values used in this work are included in Table 1. Note that the experimental values were obtained for pentapeptides, while tripeptides were used for computing  $V^{\text{MM}}$ . This however did not affect the results, as shown in Figure S2 of SI.

Thermodynamic integration was used to compute the reference free energies as follows: The partial charges in tripeptide systems representing the reference states of Glu, Asp, His were linearly interpolated between  $\lambda = -0.1$  and  $\lambda = 1.1$  with a step of 0.05 under the constraint  $\lambda_1 + \lambda_2 = 1$  for Glu and Asp, while for His, the constraint was  $\lambda_1 + \lambda_2 + \lambda_3 = 1$ .

For each set of  $\lambda$  values, called a grid point, a 10 ns MD simulation was performed.  $\partial V/\partial \lambda_i$  were saved every ps, which is approximately equal to the auto-correlation times for the  $\lambda$ -coordinates. The total charge of the system was kept neutral by simultaneously changing the charge of a single buffer particle, discussed below. The  $\partial V/\partial \lambda_i$  values were averaged over the last 9 ns of the trajectories. To obtain an analytical expression for  $V^{\text{MM}}$  a fifth order polynomial was fitted to these averages for Asp and Glu, while an eighth order polynomial was fitted for His, taking into account possible linear dependencies of the coefficients (see Supplementary Information). Fitting errors were below 0.5 kJ/mol for Asp and Glu and below 1 kJ/mol for His, which are of similar magnitude as the statistical accuracy of the derivatives.

### Buffer particles

Dynamically changing partial charges can affect the total charge of the simulation unit cell, which can lead to artifacts, as documented for instance in Hub *et al.* for Ewald-based methods.<sup>59</sup> To avoid such artifacts, it is essential to keep the total charge of the unit cell constant. Two approaches have been proposed: (i) direct coupling between each titratable residue and a water,<sup>20</sup> or ion,<sup>18</sup> and (ii) titratable buffers that collectively compensate changes in charge of all titratable residues.<sup>34</sup>

Here, we follow the latter approach, but with several improvements for all-atom simulations. Firstly, to avoid restraints, which were needed to minimize interactions between the buffers and the titratable sites in previous work,<sup>34</sup> we introduced buffer particles with both small LJ radius and small partial charges of maximal  $|0.5| e$ , such that they do not disturb the hydrogen bond network, nor interact too strongly with the titratable sites or other buffers. Secondly, to also prevent strong interactions with hydrophobic regions in the system, the C6 dispersion parameter with anything other than water was set to zero, including the other buffers. The latter also avoids clustering of buffers during the simulation. Further details on buffer parameterization are provided in the accompanying paper.<sup>46</sup> Thus, the buffer particles

have an  $\sigma$  of 0.25 nm and an  $\epsilon$  of 4 kJmol<sup>-1</sup>. In coarse-grained simulations, standard Na<sup>+</sup> ions were used as buffer particles.

As in Donnini *et al.*,<sup>34</sup> the buffers were collectively coupled to the titratable sites in the system via a charge constraint. The charges of all buffers were thus simultaneously interpolated between  $-0.5 e$  and  $0.5 e$ , keeping the simulation box neutral. For all peptide simulations, 10 such buffers were introduced into the system, while 20 buffers were added to the simulation boxes with the proteins (systems 4-5), in both AA and CG models. 185 buffer particles were used in GLIC simulations.

## Analysis of the constant pH trajectories

To estimate the  $pK_a$  values of titratable groups from multiple simulations at various pH values, we computed the average fraction of deprotonated frames ( $S^{\text{deprot}}$ ) over all replicas. For a group with a single titratable site, this average was obtained as

$$S^{\text{deprot}}(\text{pH}) = \frac{N^{\text{deprot}}}{N^{\text{prot}} + N^{\text{deprot}}} \quad (18)$$

where  $N^{\text{prot}}$  and  $N^{\text{deprot}}$  are the total number of frames in which the site is protonated and deprotonated, respectively. For titratable sites modeled in the single-site representation, we considered it protonated if  $\lambda$  is below 0.2, and deprotonated if  $\lambda$  is above 0.8. For sites that are described with the multisite description, we considered a state protonated if the  $\lambda$  associated with the protonated form of the residue is above 0.8, and deprotonated if the  $\lambda$  associated with the deprotonated form of the residue is above 0.8.

To estimate the macroscopic  $pK_a$  values of histidine, which contains two titratable sites  $N_\epsilon$  and  $N_\delta$ , we calculated for each pH value the average fraction of frames in which the residue is deprotonated at either of the two sites:

$$S_{\text{macro}}^{\text{deprot}}(\text{pH}) = 1 - \frac{N^{\lambda_p}}{N^{\lambda_p} + N^{\lambda_\epsilon} + N^{\lambda_\delta}} \quad (19)$$

where  $N^{\lambda_p}$ ,  $N^{\lambda_\epsilon}$  and  $N^{\lambda_\delta}$  are the numbers of frames in which  $\lambda_p > 0.8$ ,  $\lambda_\epsilon > 0.8$  and  $\lambda_\delta > 0.8$  (Figure 2). To estimate the microscopic  $pK_a$  values for the two sites of His, we calculated for each site the average fraction of frames in which that site was deprotonated:

$$S_{\text{micro}(\delta/\epsilon)}^{\text{deprot}}(\text{pH}) = \frac{N^{\lambda_\delta/\lambda_\epsilon}}{N^{\lambda_p} + N^{\lambda_\epsilon/\lambda_\delta}} \quad (20)$$

Errors were estimated from the standard error of the mean for the different replicas.

The averaged fractions at each pH value were fitted to the Henderson-Hasselbalch equation:

$$S^{\text{deprot}} = \frac{1}{10^{(\text{p}K_a - \text{pH})} + 1} \quad (21)$$

which yielded the  $pK_a$  values as fitting parameters. The error in the  $pK_a$  was estimated from 95% confidence interval for the non-linear least squares fit to the average ( $S^{\text{deprot}}$ ) values.

## Results and Discussion

Here we discuss the results obtained with our new implementation of constant pH into the fork of GROMACS 2021 release.<sup>40</sup>

### Titration of single amino acids

In Figure 3 we show the titration curves for AlaAspAla, AlaGluAla and AlaHisAla tripeptides, obtained from simulations with the modified all-atom CHARMM36<sup>46</sup> and coarse-grained Martini 2.0 force fields.<sup>33</sup> Fitting the deprotonated fractions as a function of pH value to the Henderson-Hasselbalch equation (dashed lines in Figure 3) yields  $pK_a$  values for the tripeptides that are within 0.1  $pK_a$  units from the reference values. Comparing the titration curves obtained with the Martini 2.0 force field in our implementation to those computed with the constant pH approach developed explicitly for this coarse-grained model,<sup>26</sup> our results suggest a much better agreement with experiment than the latter. We attribute

this difference to the more sophisticated explicit treatment of proton-like particles in the Martini constant pH approach. The rather good agreement between the titration curves obtained for both force fields on the one hand and experiment on the other hand suggests that our implementation has little to no dependency on the force field, in line with the GROMACS philosophy of supporting a wide range of popular force fields.

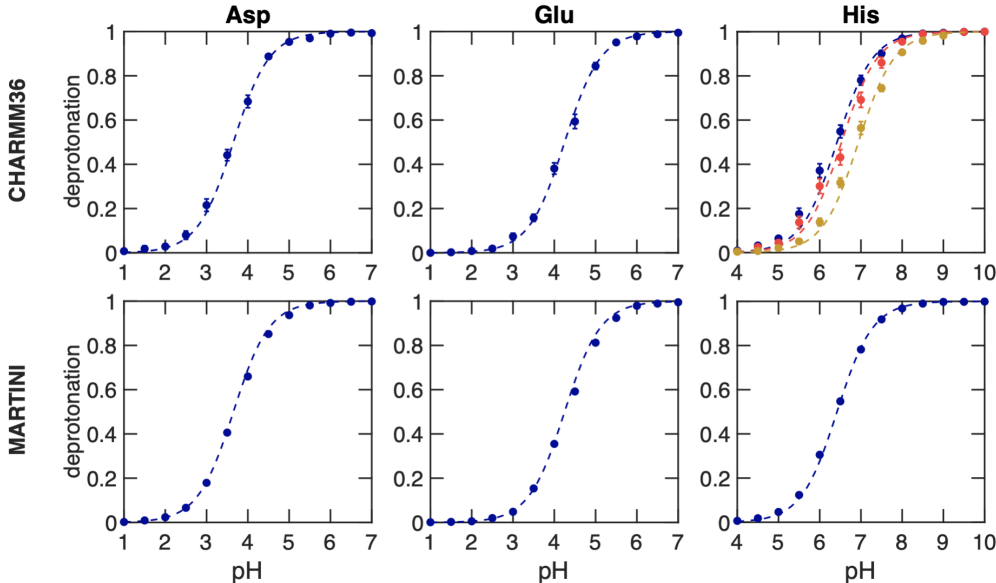


Figure 3: Titrations of tripeptide amino acids (Glu, Asp, His) in water. The top and bottom rows show titrations performed with the modified AA CHARMM36<sup>46</sup> and CG Martini<sup>33</sup> force fields, respectively. In all simulations neutrality was maintained by including ten buffer particles in combination with the charge constraint. Dots show the fraction of frames in which the residue is deprotonated, and the dashed lines represent the fits to the Henderson-Hasselbalch equation. For His, blue color represents the macroscopic  $pK_a$ , while yellow and red represent the microscopic  $pK_a$  values for HSD (proton on  $N_\delta$ ) and HSE (proton on  $N_\epsilon$ ), respectively. In the Martini 2.0 model, HSD and HSE are indistinguishable and hence only the macroscopic titration curve is shown. From the fits the  $pK_a$  values were estimated and listed in Table 1.

## Titration of proteins

The titration curves of cardiotoxin V and HEWL proteins are shown in Figures 4 and 5, respectively. The  $pK_a$  values obtained from fitting the Henderson-Hasselbalch equation to the degree of deprotonation in the all-atom simulations of both proteins with the CHARMM36



Table 1:  $pK_a$  values obtained from titration simulations. The reference  $pK_a$  values for tripeptides are given in the last column that contains the experimental  $pK_a$  values. The values for Asp and Glu are taken from,<sup>60</sup> while the microscopic and macroscopic  $pK_a$  values for His are taken from.<sup>61</sup> Experimentally obtained  $pK_a$  values for cardiotoxin V are from<sup>62,63</sup> and for HEWL from.<sup>64</sup>

Amino acid	$pK_a$ values		
	CHARMM36	MARTINI	Exp.
	Tripeptide simulations <sup>60,61</sup>		
Asp	$3.61 \pm 0.03$	$3.69 \pm 0.02$	3.65
Glu	$4.26 \pm 0.04$	$4.30 \pm 0.03$	4.25
His macroscopic	$6.34 \pm 0.08$	$6.40 \pm 0.03$	6.42
His HSD	$6.56 \pm 0.06$		6.53
His HSE	$6.90 \pm 0.05$		6.94
	Simulation of cardiotoxin V <sup>62,63</sup>		
His-4	$5.14 \pm 0.16$	$4.54 \pm 0.09$	5.5
Glu-17	$4.08 \pm 0.08$	$4.36 \pm 0.04$	4
Asp-42	$4.02 \pm 0.10$	$4.30 \pm 0.05$	3.2
Asp-59	$2.41 \pm 0.07$	$1.45 \pm 0.03$	< 2
	Simulation of HEWL <sup>64</sup>		
Glu-7	$2.82 \pm 0.07$	$4.86 \pm 0.05$	2.6
His-15	$4.84 \pm 0.05$	$5.42 \pm 0.05$	5.5
Asp-18	$3.35 \pm 0.05$	$3.31 \pm 0.03$	2.8
Glu-35	$7.64 \pm 0.13$	$6.36 \pm 0.05$	6.1
Asp-48	$0.99 \pm 0.07$	$3.36 \pm 0.05$	1.4
Asp-52	$5.69 \pm 0.12$	$7.18 \pm 0.10$	3.6
Asp-66	$1.70 \pm 0.10$	$5.22 \pm 0.05$	1.2
Asp-87	$1.73 \pm 0.03$	$3.47 \pm 0.05$	2.2
Asp-101	$5.43 \pm 0.11$	$4.20 \pm 0.06$	4.5
Asp-119	$2.77 \pm 0.05$	$3.80 \pm 0.05$	3.5

force field, listed in Table 1, are in good agreement with previous constant pH MD simulations,<sup>23,65</sup> and in reasonable agreement with experimental estimates from NMR spectroscopy.<sup>62–64</sup> In particular, the trends in the  $pK_a$  shifts are well reproduced, including the downshift of Asp-59 in cardiotoxin V, and, with the exception of Glu-35 and Asp-52 in HEWL, the deviations are below 1  $pK_a$  unit. We note that also in previous constant pH simulations with the CHARMM force field,<sup>15,23</sup> similar deviations were found for these two residues (see Figure S5). This suggests that the origin of the discrepancy might lie beyond the implementation, and could be due to either a lack of sampling, or systematic shortcom-

ings in the force field. The  $pK_a$  values estimated from the Martini 2.0 force field simulations of these proteins do not agree as well to experiment as those derived from the all-atom simulations. We speculate that the larger deviation of the  $pK_a$ 's in the coarse-grained constant pH simulations could be due to the lower accuracy of the electrostatic interactions. Although we still consider the results obtained with the Martini simulations reasonable, in particular for the peptides, the discrepancies for the titratable residues in proteins suggest that additional parameterization efforts may be required to systematically improve the force field for constant pH MD simulations based on  $\lambda$ -dynamics. Such improvements would be particularly worthwhile considering coarse grained simulations pave the way to perform MD simulations of complete organelles,<sup>66</sup> in which many processes have a strong pH dependence.

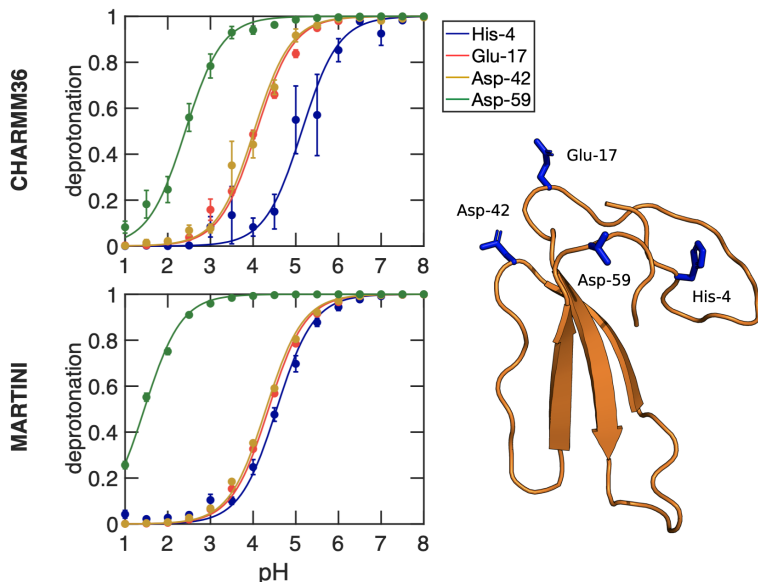


Figure 4: Titration curves of the cardiotoxin V protein obtained from constant pH MD simulations with the CHARMM36 (top) and Martini 2.0 force fields (bottom). For each of the four titratable residues in this protein the dots show the fraction of frames in which the residue is deprotonated. The lines show the best fits to the Henderson-Hasselbalch equation. The  $pK_a$  values for each titratable residue were estimated from these fits, and listed in Table 1. The right panel shows the protein structure with the four titratable residues highlighted in stick representation.

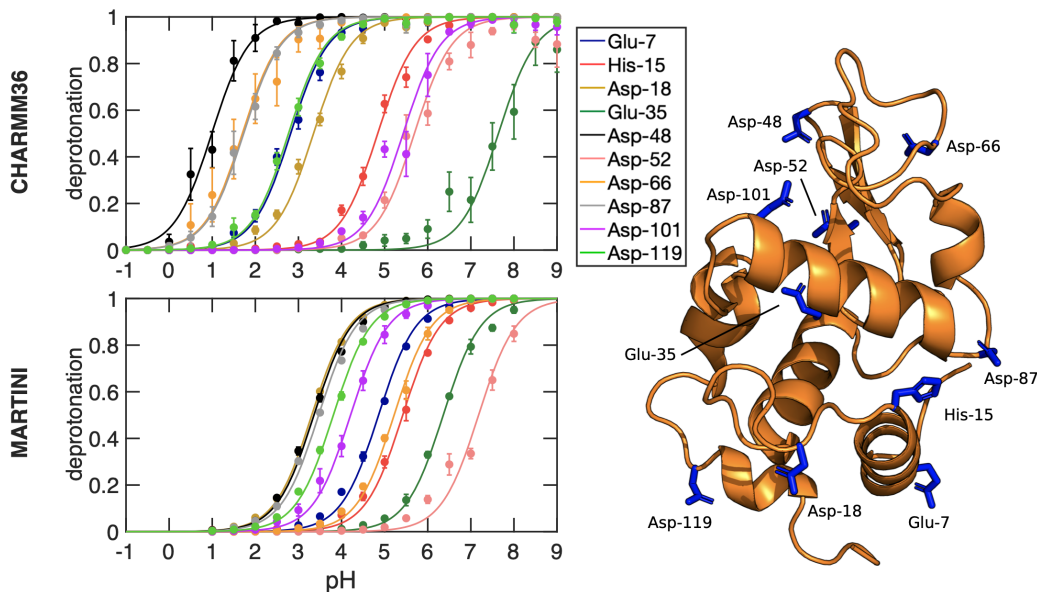


Figure 5: Titration curves of the HEWL protein obtained from constant pH MD simulations with the CHARMM36 (top) and Martini 2.0 force fields (bottom). For each of the ten titratable residues, the dots show the fraction of frames in which that residue is deprotonated. The lines show the best fit to the Hendersen-Hasselbach equation. The  $pK_a$  values for each titratable residue were estimated from these fits, and listed in Table 1. The right panel shows the protein structure with the ten titratable residues highlighted in stick representation.

## Efficiency of the implementation

To demonstrate that linear interpolation of charges is computationally more efficient than linear interpolation of the potential energy functions for systems with many titratable sites, we investigated how the computational cost of a simulation scales with the number of titratable sites in the system for both approaches. Because we have implemented interpolation of the charges rather than potentials into the fork of GROMACS 2021 release, whereas the potential interpolation was implemented in a fork of GROMACS 3.3 release, we compare the *relative* performances of both codes for an increasing number of titratable sites in the system. We define the *relative* performance as the ratio between the average wall-clock time per integration step for a simulation with constant pH on the one hand and the average wall-clock time per integration step for a normal simulation without constant pH on the other hand.

Figure 6A shows that relative performance of constant pH simulations with charge interpolation does not decrease when the number of titratable sites included in the simulation increases. Most of the 30-40% drop in performance compared to a normal MD simulation with the same version of GROMACS is caused by the additional calculations and reductions in the non-bonded pair-interaction kernels that are required to obtain the real-space part of the electrostatic potential ( $\Phi(\mathbf{R}_i, \boldsymbol{\lambda})$  in Equation 17).

In contrast, the relative performance of constant pH simulations based on the linear interpolation of potential energy functions, decreases with the number of titratable sites in the system. This comparison thus demonstrates that by replacing linear interpolation of potentials by linear interpolation of partial charges, we have overcome the major bottleneck in the earlier constant pH implementation in the fork of GROMACS 3.3 release, and paved the way towards simulations of large biomolecular systems at constant pH. An example of such a large system is the proton-gated ion channel GLIC, a membrane protein with 185 titratable residues. Figure 6B shows the performance of the new implementation for this large system when running the simulation on CPU and on a combination of CPU and GPU. While the computational overhead is somewhat larger when using a GPU in addition to a CPU, the overall performance still improves significantly when adding a GPU.

## Conclusions

We have presented and validated a new implementation of  $\lambda$ -dynamics based constant pH molecular dynamics in the GROMACS software. Our implementation combines several developments in this field into a single MD program, including the multisite representation of titratable groups,<sup>38</sup> charge interpolation,<sup>14</sup> Particle Mesh Ewald electrostatics,<sup>23</sup> and charge constraints.<sup>34</sup> Test calculations on amino acids and proteins suggest that the new implementation is efficient, accurate and agnostic to force fields. Combined with user-friendly parameterization protocols, presented in the accompanying paper,<sup>46</sup> we expect that this im-

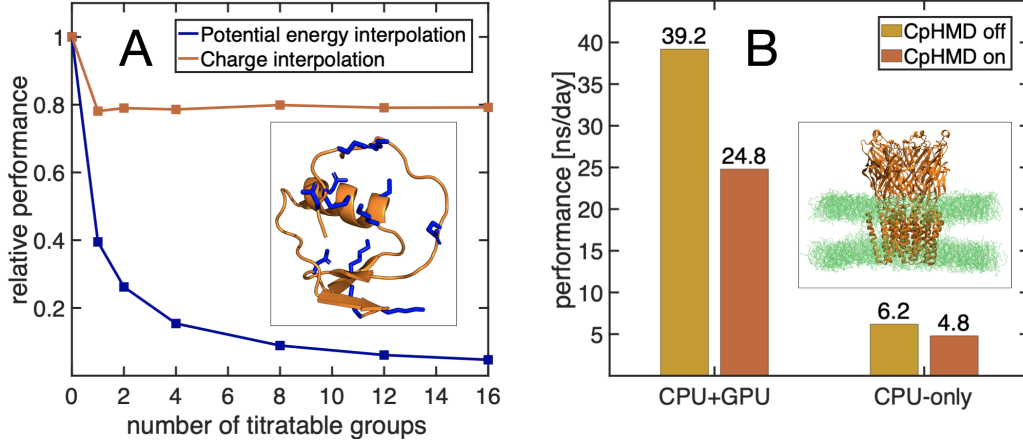


Figure 6: (A) Relative performance of interpolating potentials in a previous implementation of CpHMD into a fork of GROMACS 3.3 release (blue) and of charge interpolation in our new implementation (red) as a function of the number of titratable sites. The simulations were performed for the turkey ovomucoid inhibitor protein (PDB ID: 2GKR<sup>44</sup>), shown in the inset, where the titratable sites are highlighted in stick representation. (B) Comparison of the performance between CPU-only and CPU+GPU implementations for the ligand-gated ion channel GLIC (PDB ID: 4HFI<sup>43</sup>) with 185 titratable sites. In total, the GLIC system contained 292135 atoms.

plementation will pave the way towards routinely including the effect of pH in biomolecular MD simulations.

## Appendix A: Constraint algorithm

We use constraints to restrict sampling to the correct protonation states in the multisite representation as well as to maintain a neutral charge of the simulation box. Both multisite and charge constraints keep the linear combination of a subset of  $\lambda$  coordinates constant, and are applied simultaneously. Thus, there are  $N_c$  constraint equations

$$\sigma^k(\boldsymbol{\lambda}) = \sum_{i=1}^{N_{\text{sites}}} \alpha_i^k \lambda_i = C^k \quad (22)$$

for  $k \leq N_c$ . Here,  $\boldsymbol{\lambda}$  is the vector of all  $\lambda_i$ -coordinates and  $C^k$  is the value of constraint  $k$ , which can be zero. If  $\sigma^k(\boldsymbol{\lambda})$  is a multisite constraint,  $\alpha_i^k = 1$  for  $\lambda_i$ -coordinates that represent

one of the protonation states of a residue, while  $\alpha_i^k = 0$  for all other  $\lambda_i$ -coordinates. If  $\sigma^k(\boldsymbol{\lambda})$  is a constraint for keeping the overall charge constant,  $\alpha_i^k = \sum_j^{N_{\text{atoms}}^i} q_{j,i}^B - q_{j,i}^A$ , with  $N_{\text{atoms}}^i$  the number of atoms whose charges change as a function of  $\lambda_i$ .

During a leap-frog integration step all  $\lambda_i$ -coordinates are first propagated without constraints to their unconstrained new values  $\lambda_i^u$ , which do not fulfill the constraints in Equation 22. To obtain the constrained  $\lambda_i^c$  values, we first connect the constrained and unconstrained  $\lambda_i$  values using the definition of  $\sigma^k(\boldsymbol{\lambda})$ :

$$\sigma^k(\boldsymbol{\lambda}^c) = \sigma^k(\boldsymbol{\lambda}^u) + \sum_{i=1}^{N_{\text{sites}}} \alpha_i^k [\lambda_i^c - \lambda_i^u] \quad (23)$$

Because the unconstrained and constrained  $\lambda_i$ -coordinates are also connected by the constraint forces  $G_i^k = -\zeta^k \frac{\partial \sigma^k}{\partial \lambda_i}$ , we have in addition that

$$\lambda_i^c = \lambda_i^u + \sum_{k=1}^{N_c} G_i^k \frac{\Delta t^2}{m_i} = \lambda_i^u - \sum_{k=1}^{N_c} \zeta^k \frac{\partial \sigma^k}{\partial \lambda_i} \frac{\Delta t^2}{m_i} = \lambda_i^u - \sum_{k=1}^{N_c} \zeta^k \alpha_i^k \frac{\Delta t^2}{m_i} \quad (24)$$

where  $\zeta^k$  is the Lagrange multiplier for constraint  $k$ ,  $m_i$  the fictitious mass of  $\lambda_i$  and  $\Delta t$  the integration time step. Substituting Equation 24 in 23 yields

$$\sigma^k(\boldsymbol{\lambda}^c) = \sigma^k(\boldsymbol{\lambda}^u) - \sum_{i=1}^{N_{\text{sites}}} \alpha_i^k \sum_{l=1}^{N_c} \zeta^l \alpha_i^l \frac{\Delta t^2}{m_i} \quad (25)$$

which after rearranging can be expressed as

$$\Delta \sigma^k = \sigma^k(\boldsymbol{\lambda}^u) - \sigma^k(\boldsymbol{\lambda}^c) = \sum_{l=1}^{N_c} \zeta^l \sum_{i=1}^{N_{\text{sites}}} \alpha_i^k \alpha_i^l \frac{\Delta t^2}{m_i} \quad (26)$$

The last expression can be rewritten in matrix form

$$\boldsymbol{\Delta \sigma} = (\Delta \sigma^1, \dots, \Delta \sigma^{N_c})^T = \mathbf{A} \boldsymbol{\zeta} \quad (27)$$

where  $\boldsymbol{\zeta} = (\zeta^1, \dots, \zeta^{N_c})^T$  and

$$\mathbf{A} = \left( \sum_{i=1}^{N_{\text{sites}}} \alpha_i^k \alpha_i^l \frac{\Delta t^2}{m_i} \right) \quad (28)$$

Because the  $\alpha_i^k$  coefficients remain the same, matrix  $\mathbf{A}$  is computed once at the start of the simulation. At each step, the elements  $\Delta\sigma^k$  are evaluated as the difference between the  $\sigma^k(\boldsymbol{\lambda}^u)$  and  $\sigma^k(\boldsymbol{\lambda}^c) = C^k$ :

$$\Delta\sigma^k = \sigma^k(\boldsymbol{\lambda}^u) - C^k \quad (29)$$

The Lagrange multipliers  $\zeta^k$  are then obtained from Equation 27 and used to correct the unconstrained  $\lambda_i$  values (Equation 24).

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## Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website. Description of  $\lambda$ -potentials, effect of neglecting the interpolation of Lennard-Jones interactions, titration results for Asp and Glu within the single-site representation, a comparison of  $pK_a$  values for HEWL obtained with various  $\lambda$ -dynamics-based constant pH methods, demonstration that charge interpolation requires a single evaluation of the electrostatic potential for both single- and multisite representations, a Mathematica notebook with instructions and routines to fit  $V^{\text{MM}}$ .<sup>67</sup> The fork of GROMACS 2021 with constant pH implemented as

described here, is available for download free of charge from <https://gitlab.com/gromacs-constantph/constantph>. In addition to the source code, also instructions on how to setup and perform MD simulations are available.

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