A Projector-Embedding Approach for Multiscale Coupled-Cluster Calculations Applied to Citrate Synthase

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ABSTRACT: Projector-based embedding has recently emerged as a robust multiscale method for the calculation of various electronic molecular properties. We present the coupling of projector embedding with quantum mechanics/molecular mechanics modeling and apply it for the first time to an enzyme-catalyzed reaction. Using projector-based embedding, we combine coupled-cluster theory, density-functional theory (DFT), and molecular mechanics to compute energies for the proton abstraction from acetyl-coenzyme A by citrate synthase. By embedding correlated ab initio methods in DFT we eliminate functional sensitivity and obtain high-accuracy profiles in a procedure that is straightforward to apply.

INTRODUCTION

Electronic structure calculations are becoming essential for identifying and testing reaction mechanisms in enzymes and are finding ever wider application in biology and biochemistry. To reach accurate chemical results in biological systems a common approach is to combine a quantum mechanics (QM) treatment of the reacting center of an enzyme combined with a molecular mechanics (MM) approach for the enzyme environment. Such calculations allow for the examination of short-lived species, such as transition states and reaction intermediates, that are often not directly accessible through experimental methods.

It is known that for some enzyme reaction mechanisms Hartree–Fock (HF) theory can give barriers up to two times higher than experiment. Second-order Möller–Plesset perturbation theory (MP2) is more accurate, but tends to underestimate barriers. Density functional theory (DFT) is an attractive alternative, but its accuracy can be hard to predict, and often it is useful to validate models against coupled-cluster calculations. Reliable calculations require high-level methods: “chemically accurate” (within 1 kcal mol⁻¹ of experiment) predictions of reaction barriers require coupled-cluster theory. Such accurate QM calculations are possible using local coupled cluster methods (e.g., LCCSD(T0)). An alternative is to use (nonlocal) spin-component-scaled MP2 (SCS-MP2) in QM/MM calculations, as this method correlates reasonably well with LCCSD(T0) results for reaction barriers, often compensating for the underestimation of MP2.

DFT is successfully used as an efficient QM method in many research communities, but picking the “right” approximate exchange-correlation functional can be problematic, and many popular approximations lead to considerable errors in calculated reaction barriers. Despite ongoing improvements to density functional approximations, the choice of functional can strongly affect the calculated result. While in some cases DFT errors may be limited to underestimation of energy barriers for reactions, in others, barriers may be overestimated. In some cases, DFT with many standard functionals leads to qualitatively incorrect conclusions, for example by predicting the wrong mechanism for some enzyme-catalyzed reactions. DFT can also give poor prediction of spectroscopic properties in transition-metal complexes, such as spin-state and zero-field splittings, and g-tensors. Ab initio methods have the potential to be more accurate.

In contrast to ab initio wave function methods, no scheme for systematic improvement of DFT has been found. Most functionals lack an inherent description of van der Waals interactions arising from dispersion (although various corrections are available to overcome this; the most commonly used is the empirically corrected B3LYP-D and related variants). Inclusion of dispersion effects has been shown to improve DFT results for modeling enzyme-catalyzed reactions. Also, the self-interaction error in DFT can result in unphysical delocalization of orbitals. Ab initio theories remove ambiguity in the choice of functional and improves the predictive power of QM/MM reaction calculations. Ideally, approaches to remove such ambiguity should fit in with standard workflows and have a low overhead for adoption.

For large systems such as proteins even the relatively low scaling of DFT or local correlation methods becomes a problem, although linear scaling implementations are avail-

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Two routes around this are possible: the system can be truncated into a model system with the assumption that the removed moieties do not affect the electronic structure, or one can utilize a multiscale approach that is capable of combining speed and accuracy with the aim of capturing the majority of long-range effects. The most common multilevel approach is the QM/MM approach pioneered by Karplus, Warshel, and Levitt.\textsuperscript{1,3,41,42} Many applications of this approach in biomolecular systems use DFT in a subsystem of chemical significance for the highest accuracy energy calculations, while the rest of the system is treated with a low-cost MM empirical “force field”. The coupling between the regimes is typically achieved by polarization the QM one-electron Hamiltonian by the MM point charges and using (MM) Lennard-Jones terms for van der Waals interactions.\textsuperscript{1,42} This simple QM/MM approach can give good results for biomolecular interactions\textsuperscript{63} and reactions.\textsuperscript{5} It can be necessary to optimize Lennard-Jones parameters for QM/MM interactions\textsuperscript{64} and the consistency and compatibility of QM and MM methods should be considered.\textsuperscript{45}

In some cases artifacts can arise in QM/MM, such as overpolarization due to the point charge approximation, although newer force fields are actively being developed.\textsuperscript{46−48} While it is common to check that answers are converged with respect to the size of the quantum subsystem,\textsuperscript{49} it does not guarantee that the quantum method has captured the underlying chemistry.\textsuperscript{5,25,50} We aim to address this by highlighting a method to calculate high-level quantum chemical properties, which also provides an accurate description of the environment through quantum polarization that is obtained from standard DFT.

Wave function (\textit{ab initio}) methods allow for precise conclusions to be drawn about mechanistic pathways and spectroscopic assignments. Another attractive feature is that wave function methods allow for an easy interpretation of the sources of error due to their systematic improbability. Wave function methods such as the complete active space self-consistent field (CASSCF) method are able to tackle strongly correlated problems such as when a near degeneracy of the HOMO−LUMO (highest occupied molecular orbital−lowest unoccupied molecular orbital) occurs (i.e., bond stretching and transition metal complexes), which are usually out of the reach of DFT.\textsuperscript{58} The cost of utilizing accurate wave function methods such as the gold standard CCSD(T) is that they scale poorly with system size ($N^6$). While approximations exist that take advantage of the local nature of correlation to significantly improve scaling,\textsuperscript{51−53} it is attractive to have a method that can return accurate properties for all post-HF methods from MP2 all the way up to full configuration−interaction without strongly scaling cost with the size of the surrounding environment.

Several methods exist that are forms of quantum embedding: a simple example is the frozen core approximation for wave function correlation.\textsuperscript{64} Embedding methods can be constructed using the one-particle density matrix,\textsuperscript{55,56} Green’s functions,\textsuperscript{57} or, in a DFT formalism, the electronic density.\textsuperscript{58−60} An alternative is to use region-based local coupled-cluster methods.\textsuperscript{64}

This work focuses on the use of a conceptually simple and computationally cheap embedding technique that uses projection operators to exactly embed high-accuracy wave function methods inside DFT potentials, here called WF-in-DFT; further we incorporate this embedded treatment of the active site into an MM treatment of the wider protein environment. This multilevel approach enables us to utilize the most accurate wave function methods for chemically critical regions (subsystem $A$; see Figure 1), more approximate DFT methods (subsystem $B$) for chemical moieties that are not directly involved in the reaction but that can affect the electronic structure of subsystem $A$, and MM for the longer range effects from the protein environment (subsystem $C$).

For this study, we examine a well-characterized enzyme-catalyzed reaction, which has become a testbed for computational modeling,\textsuperscript{79,65−67} namely the deprotonation of the acetyl coenzyme A (acetyl-CoA) in citrate (Ser-)synthase. Overall this reaction forms citric acid from acetyl-CoA and oxaloacetate, the first step of the citric acid cycle. Citrate synthase is crucial for most forms of life, and it has been studied extensively both experimentally\textsuperscript{68−71} and computationally.\textsuperscript{79,65−67} The first step in the reaction mechanism of citrate synthase involves proton abstraction from the α-carbon of acetyl-CoA by an aspartate
residue (Asp375, in the numbering for the pig enzyme). The enolate intermediate that is thus formed then performs a nucleophilic attack on the carbonyl carbon of oxaloacetate, forming a stable citryl-CoA intermediate. Finally, hydrolysis of the thioester bond produces citrate and coenzyme A. For efficient catalysis of the overall reaction, stabilization of the enolate intermediate by the enzyme active site is crucial. Here, we examine the performance of WF-in-DFT calculations coupled to molecular mechanics calculations, leading to unprecedented canonical CCSD(T)-level QM/MM calculations on an enzyme catalyzed reaction.

**THEORY**

To embed wave function methods in DFT, we utilize a projector-based embedding scheme that has the property that the DFT-in-DFT (e.g., PBE-in-PBE) energy is the same as the full DFT calculation. This is achieved without having to resort to numerically challenging, iterative procedures such as optimized effective potentials. A further advantage of this method is that it only requires modification of the core Hamiltonian, so practically any wave function method can be used in the high-accuracy subsystem. The total electronic density ($\rho$) can be directly partitioned into subsystem contributions as

$$\rho^T = \rho^A + \rho^B$$

The DFT energy decomposes into contributions from the two subsystems and a nonadditive component that describes the interaction between them:

$$E[\rho^T] = E[\rho^A] + E[\rho^B] + \delta E[\rho^A, \rho^B]$$

The nonadditive component is made up of the nonadditive Coulomb, kinetic, and exchange-correlation energies; of these terms, the kinetic energy is the most challenging contribution. Although methods exist to calculate this term, they are either expensive or not yet accurate enough to be used for partitions that cut covalent bonds.

In projector-based embedding, the requirement to calculate the kinetic energy contribution is avoided by constructing the subsystem densities from orthogonal subsets of orbitals; thus, the nonadditive kinetic energy interaction is exactly zero. In our implementation, orthogonality is achieved by applying a projection operator that elevates all subsystem B orbitals to high energy, making them unavailable to the electrons of subsystem A. The subsystem-A core Hamiltonian is given by

$$h^{A\text{in}B\text{in}} = h + J[\rho^A, \rho^B] - J[\rho^A] + v_{ex}[\rho^A, \rho^B] - v_{ex}[\rho^A] + \mu \Phi^B + b_{\text{QM/MM}}^C$$

where $h$ is the original core Hamiltonian; $J[\rho^A, \rho^B] - J[\rho^A]$ describes the Coulomb interaction with electrons of subsystem B; $v_{ex}[\rho^A, \rho^B] - v_{ex}[\rho^A]$ similarly describes the exchange correlation interaction; $\mu$ is a large, positive parameter; and $b_{\text{QM/MM}}^C$ is the usual Hamiltonian describing electrostatic interaction with the MM charges in subsystem C.

The total energy for a WF-in-DFT calculation is given by

$$E[\psi^A; \rho^A, \rho^B] = (\psi^A h^{A\text{in}B\text{in}} C \psi^A) + tr \left( v_{ex}[\rho^A, \rho^B] - v_{ex}[\rho^A] \right) + E_{\text{xc}}[\rho^A, \rho^B] - E_{\text{xc}}[\rho^A] + E[0, \rho^B] + E_{\text{coupling}}^{C\text{QM/MM}} + E_{\text{MM}}^C$$

where $h^{A\text{in}B\text{in}} C$ is the embedding Hamiltonian in eq 4 used by the high-level wave function method; the central terms describe a correction for exchange-correlation double-counting; and $E_{\text{coupling}}^{C\text{QM/MM}}$ and $E_{\text{MM}}^C$ are the QM/MM contributions from subsystem C.

The canonical Kohn–Sham orbitals tend to be delocalized over the system, so to define subsystems the orbitals are first localized and then automatically assigned to atomic centers by population analysis. Previous work has shown that the intrinsic bond orbitals (IBOs) of Knizia provide reliable and chemically meaningful localized orbitals.

To substantially reduce the size of the virtual space in the WF calculation on subsystem A, we also use basis truncation to reduce the number of two-electron integrals (and wave function amplitudes) in a systematic and controllable fashion. Asymptotically, this leads to the computational cost of the high-level calculation in subsystem A becoming independent of the size of the environment. Two truncation methods are available: we use the form proposed by Bennie et al. A single parameter based on the net Mulliken population is used to screen atomic orbitals; for various chemical systems we were able to achieve submillihartree truncation errors with the threshold set to $10^{-6}$.

The procedure for CCSD(T)-in-DFT/MM requires only the definition of atomic centers in the active (A) subsystem as an extra step in the input. Overall, the procedure takes the following form:

1. Take a molecular structure (e.g., previously optimized with QM/MM).
2. Perform single point DFT on the QM subsystem (A+B).
3. Localize the orbitals.
4. Select the atoms to be in subsystem A for the high level (e.g., CCSD(T)) calculation.
5. Perform high-level (e.g., CCSD(T)) calculations on subsystem A, using the embedding Hamiltonian given in eq 4, which results in the embedded WF-in-DFT/MM energy.

**METHODS**

To study the proton abstraction of acetyl-CoA by Asp375 in citrate synthase with projector embedded QM/MM, we used previously optimized QM/MM geometries (at the B3LYP/6-31+G(d)//CHARMM27 level). These were obtained by employing a reaction coordinate defined as RC = $d(C_{\text{Acetyl-CoA}}-H) - d(O_{\text{Asp375}}-H)$. This reaction coordinate has been shown to represent the reaction pathway accurately (e.g., by comparison to the nudged elastic band method).

We used geometries that were obtained between RC = −1.4 Å and RC = 1.4 Å with a step size of 0.1 Å. For the majority of the calculations, the QM region consisted of the methylthioester part of acetyl-CoA, the side chain of Asp375, and oxaloacetate (OAA) (see the right-hand side in Figure 1). We also performed calculations with larger QM regions (regions 2–4 in Figure 4). Single-point QM/MM and WF-in-DFT/MM calculations were performed using the Molpro 2015.1 software.
package. The MM energy and QM/MM van der Waals contributions were taken from the previous B3LYP/6-31+G(d)//CHARMM27 calculations.7

All the calculations in this work used the aug-cc-pVDZ or aug-cc-pVTZ basis sets for all atoms in subsystems A and B. QM/MM calculations were performed using the LDA, PBE, B3LYP, and BH&HLYP functionals, as well as HF theory, for the QM subsystem. WF-in-DFT/MM calculations used each of these mean-field methods for subsystem B and CCSD(T), MP2, SCS-MP2, and CCSD levels on subsystem A.

The smallest QM region size we consider here is the equivalent of "region 2" in the previous high-level QM/MM work; we designate it as our "region 1". This selection was made in order to have a sufficient region to show the effect of the choice of the functional: here the oxaloacetate molecule is included in subsystem B because QM treatment of oxaloacetate has been shown to influence the reaction profile significantly. Region 1 is then subdivided into subsystems A and B, representing the CCSD(T) and DFT subsystems.

When choosing the size of subsystem A in embedding calculations it is important to ensure that enough electrons are included to obtain an accurate description of WF correlation in the reacting moiety. In the present citrate synthase case we use a selection that we have found to be widely applicable: subsystem A consisted of the three atoms directly involved in the reaction, plus all atoms up to two bonds away from these. This region is shown in Figure 1 in red, and contains 72 electrons, with the sulfur valency satisfied in the embedded calculation by including the covalent bond with the methyl group. The 1s, 2s, and 2p electrons on the sulfur atom were treated as core electrons and were not correlated in the post-HF methods. All other electrons, including the 1s electrons on carbon, nitrogen, and oxygen atoms, were correlated. The basis-set truncation scheme uses a threshold to determine how many basis functions to discard. With the threshold set to 0 the entire basis is retained, and as the threshold is increased, more subsystem B functions are discarded. The threshold values used for this study were $10^{-4}$ and $10^{-5}$. All timing comparisons were conducted in serial on a dedicated workstation with 32GB of RAM. Density plots were generated by the Molekel 5.4 program with an isovalue of 0.05. Enzyme geometries were visualized using the PyMOL software package.

## RESULTS AND DISCUSSION

Previous LCCSD(T0) QM/MM calculations indicate that the potential energy barrier for the proton abstraction in this structure is 11 kcal mol$^{-1}$ at the complete basis set limit. The transition state was found to be between 0.2 and 0.3 Å along the reaction coordinate. Although the oxaloacetate is merely a spectator in the enolate formation, it has a strong effect due to its proximity, and its inclusion in the QM region was found to be essential (treating it at only the MM point charge level leads to an increase in barrier height of ~3 kcal mol$^{-1}$).66

We first explore the reaction barrier and reaction energies using various traditional methods. Our selection is broad and includes the rather primitive local density approximation (LDA) functional, a general gradient approximation functional (PBE), two hybrid functionals (B3LYP and BH&HLYP), as well as HF theory. The resulting energy profiles vary significantly (Figure 2A) in line with previous work. For example, a barrier of 20 kcal mol$^{-1}$ was found when using HF while LDA gives a small barrier of 2.5 kcal mol$^{-1}$. Pure DFT calculations underestimate the barrier and do not strongly indicate a minimum for the enolate intermediate. BH&HLYP is 4 kcal mol$^{-1}$ closer to the HF answer compared to B3LYP, presumably due to the higher proportion of exact exchange (50% vs 20%).

Using projector embedding to perform CCSD(T) in subsystem A (Figure 2B) largely removes the variation due to choice of functional in subsystem B. Across all methods the energy profiles are now quantitatively similar, particularly for the (approximate) activation energy, which has a maximum variance of 1 kcal mol$^{-1}$ between B3LYP and HF. Variation for the reaction energy of the enolate intermediate with respect to the reactant state is somewhat larger. This variance is due to HF and LDA, which may be producing poorer oxaloacetate densities, and thus failing to model the stabilization of the CoA by the oxaloacetate ketone. The GGA and hybrid functionals (PBE, B3LYP and BH&HLYP) are in much closer agreement.
for the reaction energy, with a variation of under 0.4 kcal mol\(^{-1}\), compared to 6 kcal mol\(^{-1}\) without embedding. Notably, the locations of the reactant state minima, the transition state maxima, and the enolate intermediate minima are very independent of functional in the embedding calculations (reaction coordinate values of \(-1.0\), 0.3, and 1.0 Å, respectively). This contrasts to the results without embedding, with which widespread values are found for the reactant minimum, between \(-1.3\) (HF) and \(-0.4\) Å (LDA), and for the product minimum, which ranges between 0.6 and 1.1 Å. This again highlights the consistency of projector-based WF-in-DFT embedding compared to traditional DFT.

Previously, LCCSD(T0)/aug-cc-pVDZ/MM energy calculations with the same geometries (with LCCSD(T0) employed for subsystems A and B) indicated an activation barrier of 13.2 kcal mol\(^{-1}\) and a reaction energy of 8.4 kcal mol\(^{-1}\). Here, CCSD(T)-in-DFT calculations with all functionals for the environment were found to be within 2 kcal mol\(^{-1}\) of LCCSD(T0) for the activation barrier, and the calculations using PBE, BH&HLYP, and B3LYP were within 1 kcal mol\(^{-1}\) for the reaction energy. The CCSD(T)-in-DFT methods resulted in a barrier of between 11.4 and 12.1 kcal mol\(^{-1}\). This includes CCSD(T)-in-LDA, which in sharp contrast to the unembedded LDA result has both the correct qualitative shape for the transition state as well as barrier height. The HF method resulted in a barrier 0.3–1.0 kcal mol\(^{-1}\) higher than obtained with the density functionals. A contributing factor to this difference is probably that the underlying HF method lacks correlation and therefore provides a poorer embedding environment; this is also reflected in the HF barrier being a factor of around two too high in Figure 2A. Using B3LYP and BH&HLYP as the subsystem B method resulted in reaction profiles that differed by a maximum of 0.1 kcal mol\(^{-1}\), showing that the density from a reasonable functional results in almost identical barriers, despite the intrinsic difference between these two functionals being up to 4 kcal mol\(^{-1}\).

The basis truncation default of \(10^{-4}\) reduced the number of system basis functions from 517 contracted functions to 412, which corresponds to 62% of the environment (Figure 2B). The looser threshold (\(10^{-3}\)) retains 352 functions, corresponding to 40% of the environment (Figure 2C). We find little effect on the barrier height despite a reduction in computational cost by a factor of 2 (averaged over the profile). It should be noted that the enolate minimum energy differs by, at most, 1.2 kcal mol\(^{-1}\) for HF, but for PBE and B3LYP is only 0.2 kcal mol\(^{-1}\) higher compared to the tighter truncation threshold of \(10^{-4}\). Calculations with a looser threshold (such as \(10^{-3}\)) may be useful to explore the performance of WF-in-DFT/MM calculations, but it is advisable to use an energetically tighter threshold (such as the default \(10^{-4}\)) for final results.

Projector embedding only requires a modified core Hamiltonian, so it is possible to use essentially any electronic structure method in subsystem A. We can thus compare how various approaches to include electron correlation in subsystem A affect the reaction energy profile (Figure 3; PBE is used in subsystem B). As expected, the absence of electron correlation (HF) leads to the largest barrier of 20 kcal mol\(^{-1}\). It should be noted that HF embedded in PBE (Figure 3) leads to a different profile than unembedded HF (Figure 2A), this is because HF is “restrained” by the PBE environment, leading to a decrease of the barrier height of 1.6 kcal mol\(^{-1}\). When taking CCSD(T) as the reference, MP2 underestimates the activation and reaction energies by 1.7 kcal mol\(^{-1}\) and 1.0 kcal mol\(^{-1}\), respectively.

Spin-component-scaled MP2 (SCS-MP2) is more accurate with activation and reaction energies 0.6 kcal mol\(^{-1}\) and 0.1 kcal mol\(^{-1}\) higher than CCSD(T), respectively. The relative performance of MP2 and SCS-MP2 compared to CCSD(T) is consistent with what was found previously for the same reaction and the two other enzyme reactions studied at this level,\(^{112}\) with (L)MP2 underestimating activation barriers and SCS-(L)MP2 showing activation barriers close to the LCCSD(T0) result. Increasing the basis size from aug-cc-pVDZ to aug-cc-pVTZ was found to decrease the barrier height by 0.1–0.3 kcal mol\(^{-1}\) and to move the transition state to RC = 0.2 Å for all methods other than HF.

Previous work by Van der Kamp et al. showed the SCS-MP2 results to be insensitive to the basis size, while the LCCSD(T0) results were found to be more strongly affected by the size of basis, changing by up to 1.5 kcal mol\(^{-1}\) with aug-cc-pVTZ. Our results here agree with the canonical SCS-MP2 of that work, in that the basis size does not have a large effect on barrier height. It is possible that the effect of increasing basis set size found previously for LCCSD(T0) calculations may result from a coupling between domain completeness and basis-set size. It has been shown that the basis dependence of LCCSD(T0)/aug-cc-pVTZ calculations of activation barriers for the hydroxylation reaction catalyzed by p-hydroxybenzoate hydrogenase can be circumvented by performing LCCSD/aug-cc-pVTZ with a coupled cluster triples correction in the smaller (aug)-cc-pVDZ basis but with the same domains as in the larger basis.\(^{112}\) It may thus be “good practice” to use a large basis with older domain-based local correlation methods to avoid an artificially high barrier due to basis-set incompleteness effects, or more admirably newer local methods with F12 explicit correlation should be used.\(^{109–102}\)

Truncated projector embedding has the attractive property that the cost of the coupled-cluster calculation is not (formally) linked to the size of subsystem B, provided that the active subsystem A density does not become more delocalized over the system as more fragments are added to subsystem B. The decoupling occurs because the number of atomic functions needed to describe the molecular orbitals in the active subsystem becomes constant as atomic functions on increasingly distant atoms are added. Figure 4 shows that for the smallest region (encompassing the methylthioester part of

![Figure 3. WF-in-DFT/MM potential energy profiles of the proton transfer from acetyl-CoA to Asp375 in citrate synthase: Effect of different WF methods. In all cases, the WF method is embedded in a PBE environment. Left: aug-cc-pVDZ basis on all atoms. Right: aug-cc-pVTZ basis on all atoms.](Image 334x593 to 555x749)
Once the region grows to encompass over ∼800 basis functions, the lack of coupling to the environment size means that embedding remains moderate in cost. When using full triples, the local coupled cluster (DF-LCCSD(T)) calculation has a much higher cost.

Despite the fact that embedding provides a reasonable degree of decoupling between the active subsystem and the DFT environment, some atomic orbitals from neighboring atoms are needed, both for flexibility in the active subsystem wave function, and for proper representation of the projection operator. This effect can be minimized by using larger basis sets in the active subsystem or by neglecting diffuse functions in the environment.

The right-hand side plot of Figure 4 shows the effect when neglecting the triples and doing the embedded CCSD calculation with direct integral evaluation. Changing from the density fitted (104–108) LCCSD (dashed green) to the full 4-index integrals (dashed orange) causes a ∼10-fold increase in cost for the local correlation method. These results indicate that a similar speedup would be possible if one was to embed a full perturbative treatment of triple excitations (T). Once the region grows to encompass over ∼800 basis functions, the lack of coupling to the environment size means that embedding remains moderate in cost. When using full triples, the local coupled cluster (DF-LCCSD(T)) calculation has a much higher cost.

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Author Contributions
The work to couple QM/MM with projector embedding was initiated by S.J.B. and M.W.v.d.K., embedding calculations were performed by S.J.B., R.C.R.P. and M.S. The initial programming to couple the MM potential to projector embedding was done by S.J.B. and M.S. All authors contributed to the analysis of results and the writing of the manuscript and have given approval to the final version of the manuscript. Note: All data from this work are held in an open-access repository.  

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Notes
The authors declare no competing financial interest.

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