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Maksym Soloviov and Markus Meuwly

Department of Chemistry, University of Basel, Klingelbergstrasse 80, 4056 Basel, Switzerland

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The transfer of CO from heme $a_3$ to the Cu$_B$ site in Cytochrome c oxidase (CcO) after photolysis is studied using molecular dynamics simulations using an explicitly reactive, parametrized potential energy surface based on density functional theory calculations. After photodissociation from the heme-Fe, the CO ligand rebinds to the Cu$_B$ site on the sub-picosecond time scale. Depending on the simulation protocol the characteristic time ranges from 260 fs to 380 fs which compares with an estimated 450 fs from experiment based on the analysis of the spectral changes as a function of time delay after the photodissociating pulse. Following photoexcitation $\approx$90% of the ligands are found to rebind to either the Cu$_B$ (major component, 85%) or the heme-Fe (minor component, 2%) whereas about 10% remain in an unbound state. The infrared spectra of unbound CO in the active site is broad and featureless and no appreciable shift relative to gas-phase CO is found, which is in contrast to the situation in myoglobin. These observations explain why experimentally, unbound CO in the binuclear site of CcO has not been found as yet. © 2014 AIP Publishing LLC. [http://dx.doi.org/10.1063/1.4870264]

I. INTRODUCTION

The dynamics of small ligands in proteins strongly depends on their environment. One of the routinely employed probe molecules is carbon monoxide (CO) which is often used in ligand binding-rebinding experiments. In such experiments, the ligand is dissociated from its binding partner, which is typically the iron atom of a heme group, by using a UV/visible laser pulse that promotes the system to an electronically excited and repulsive state. Subsequently, the time-resolved data of the probe molecule allows to follow the time-varying environmental changes by spectroscopic means. The structural interpretation of the data, including the nature of the electrostatic environment, the ligand migration pathways and reactivities, or the ligand-protein coupling is, however, far from straightforward. Usually, the conclusions that can be drawn from experiment alone about the protein environment are indirect except for circumstances under which spectroscopic and structural signatures can be recorded simultaneously which is only possible in rare cases. Alternatively, validated molecular dynamics (MD) simulations provide the required temporal and spatial resolution to complement experimental investigations and allow to interpret the dynamics at atomic resolution.

The ultrafast dynamics of cytochrome c oxidase (CcO) has previously been investigated using a number of experimental techniques which provide information about the protein environment and ligand binding-rebinding reactions. The physiological function of CcO is to reduce molecular oxygen to water. The protein contains two $a$-type heme groups: heme $a$ (low spin) is hexacoordinated and mediates electron transfer from exogenous cytochrome $c$ towards the active site whereas heme $a_3$ (high-spin), near the copper atom Cu$_B$ $\approx$5 Å away from the iron atom of heme $a_3$, acts as the binding site for molecular oxygen and its reaction intermediates during four-electron reduction. The electron equivalents are provided by cytochrome c in the outside of the mitochondrial inner membrane (the intermembrane space) via a copper site (Cu$_A$) and a low-spin heme (heme $a$) to the O$_2$ reduction site. The protons used for water formation from O$_2$ are transferred from the inside of the mitochondrial inner membrane (the matrix space) through two hydrogen-bonded networks known as the K and D pathways. In addition to O$_2$, heme $a_3$ also binds other diatomic ligands, including nitric oxide (NO) or carbon monoxide (CO) which are physiologically generated and act as biological messengers. Because heme binds CO tightly, its accessibility to the heme $a_3$ in CcO must be well controlled.

The active site of interest for the current study is the one where the four electron reduction takes place. It contains heme $a_3$, bound to the protein via histidine His$_{411}$, and a three-histidine-coordinated Cu center (Cu$_B$) located 4.5–5.0 Å away from the Fe of heme $a_3$. Together they form a bimetallic active site. Before binding to heme $a_3$, ligands such as O$_2$ and CO bind to Cu$_B$ immediately. The reverse reaction, involving ligand transfer from heme $a_3$ out of the protein via Cu$_B$, can be studied using flash photolysis. CO is known to permanently inhibit CcO by forming a thermodynamically stable complex with the heme $a_3$-Fe. As in other protein environments, the frequency of the CO stretch is sensitive to the changes in the chemical environment. This property has been extensively used in numerous spectroscopic studies of enzymatic active sites as a probe. In CcO, the CO molecule is known to bind transiently to the Cu$_B$ site before forming a thermodynamically more stable complex with the heme $a_3$-Fe. The reverse reaction can be induced by photolysis of the Fe–CO bond. Subsequent reactions involve the transfer of CO to the Cu$_B$ site which occurs on the 1–2 ps time scale.
In order to better understand the atomistic details underlying CO transfer dynamics in the bimetallic center of CcO, MD simulations together with a parametrized force field are employed. Such simulations are capable of characterizing the ligand transfer trajectories and relate structural and temporal aspects of the process. One of the open questions is whether free CO is formed at all and on what time scales it is expected to exist under such circumstances. One suitable observable to compare with is the infrared spectroscopy of CO which changes characteristically between metal-bound and free CO. Furthermore, it is of interest to explicitly follow the ligand unbinding and rebinding dynamics and to determine the typical time scales for this process which is expected to be on the sub-picosecond to picosecond time scale. This makes the present system particularly relevant for direct comparison between experiment and simulation because extensive averaging is possible.

II. METHODS

A. Molecular dynamics simulations

All simulations of Cytochrome C oxidase with bound and unbound CO were carried out using CHARMM\textsuperscript{21} with the CHARMM22 force field.\textsuperscript{22} For CcO, the X-ray structure with the Protein Data Bank reference 1AR1\textsuperscript{23} was used. The computational model of the protein contains subunits A (529 amino acids) and B (252 amino acids), 3 copper atoms, 2 heme groups (heme $a$ and heme $a_3$), calcium and magnesium ions, 9 molecules of lauryldimethylamine oxide, the carbon monoxide molecule, and 42 water molecules, which have been found in the X-ray structure. For simulations with Fe-bound CO of heme $a_3$, the initial Fe–C(CO) bond length was set to 1.90 Å. The crosslink between His$_{276}$ and Tyr$_{280}$ (N$_4$–C$_{6}$) known for bovine CcO\textsuperscript{23,24} has been introduced via a CHARMM patch. The two copper atoms, which are not part of the active site of interest are treated by applying harmonic constraints to the bond lengths and the angles between these copper atoms and the protein. The 1AR1 structure was used as this was also the reference structure in previous computational work.\textsuperscript{20} The active site structures of unligated, reduced CcO of $P$. denitrificans (1AR1), $Th$. thermophilus (3EH5), and $Bos$ $taurus$ (2EII) are structurally very similar (see Figure 1 in the supplementary material\textsuperscript{36}).

Hydrogen atoms were added using CHARMM and the protein was solvated in a pre-equilibrated water box ($80 \times 117 \times 74$ Å$^3$) which leads to a total system size of 58 092 atoms. The protonation states of the residues were determined using PROPKA\textsuperscript{25–28} and Karlsberg+.\textsuperscript{29,30} For the most relevant active site residues His$_{25}$, His$_{326}$, His$_{341}$, the standard $\delta$-protonation state was found. However, it is worthwhile to mention that “standard” protonation states can be misleading as for the His$_{64}$ residue in Myoglobin which has been shown to be $\epsilon$-protonated based on computations and experiments.\textsuperscript{31,32} All simulations used periodic boundary conditions and the TIP3 model for water.\textsuperscript{33} The total charge of the system is $-4.0e$ and was neutralized by replacing random TIP3 waters by potassium ions. Some water molecules were replaced by potassium and chloride ions so that the concentration of KCl in the water box was $\approx 0.15$ M. The SHAKE algorithm\textsuperscript{34,35} with a tolerance of $10^{-6}$ was applied to all bonds which included hydrogen atoms. A time step of 1 fs was used, and the non-bonded interactions were cut off at 14 Å. First, the system was minimized with the steepest descent algorithm, then heated to 300 K during 300 ps. The early equilibration was a 300 ps $Np$  simulation (Nose-Hoover thermostat with coupling constant 5.0 ps$^{-1}$) which leads to a pressure of 1 atm at the end of the first equilibration phase. Following this, the box size was adjusted and a 700 ps $NVT$ equilibration was run. Then, production runs were carried out for 5 ns, during which the initial configurations for the excitation were collected every 10 ps.

B. Intermolecular interactions

The global PES $V(\vec{X})$ is a function of the coordinates $\vec{X}$ of all atoms involved. For the particular case of CO transfer between the bimetallic site in CcO, we write the total energy as

$$V_{tot}(\vec{X}) = V_{FF}(\vec{Q}) + V(r, R, \theta).$$ \hspace{1cm} (1)

The force field $V_{FF}(\vec{Q})$ is the standard CHARMM22 force field and $\vec{Q}$ contains all configurational coordinates of the system except $R$, $\rho$, and $\theta$ (see below) which describe the coordinate dependence of the PES involving the binuclear site and the ligand in the electronic ground state, see Figure 1. The coordinates of the subsystem are $R = |\vec{R}|$ the distance between the Fe atom of heme $a_3$ (labeled Fe$_{a3}$ in the following) and the Cu$_8$ site, the vector $r = |\vec{r}|$ between Fe$_{a3}$ and the center of mass (COM) of CO, and $\theta$ which is the angle between $\vec{r}$ and the vector along the CO-bond. Thus, $\theta$ corresponds to the CO-rotation. In the ligand-bound state, the CO stretching coordinate was parametrized as a harmonic potential with a force constant of 1333.1 kcal/mol and an equilibrium separation of 1.128 Å, the charges are $+0.021$ and $-0.021$ for C and O, respectively.\textsuperscript{36,37}

For the three-dimensional PES $V(r, R, \theta)$, more than 3300 reference energies were calculated using density functional theory (DFT) calculations with the B3LYP\textsuperscript{38–41} functional and the 6-31G(d,p) basis set.\textsuperscript{42,43} All electronic

![FIG. 1. (a) The model of the system (red – active site of interest, cyan – protein backbone. (b) Diagram showing variables of the potential.](image-url)
structure calculations were carried out with Gaussian09.44 For the electronic structure calculations, a grid was defined which includes Fe–Cu distances between 4.3 and 5.5 Å with a spacing of 0.1 Å, Fe–CO(CoM) distances from 1.5 to 3.5 Å and angles θ corresponding to an 11-point Gauss-Legendre grid. Such a definition for the angular grid was found to enhance the stability of the fit and allow for a convenient representation of the angular degree of freedom in fitting the three-dimensional PES.53–58 We found it convenient to introduce the dimensionless coordinate ρ defined as ρ = \frac{r - \rho_{eq}}{\sigma} where σ = 1.5 Å is the minimal Fe–C and Cu–C distance encountered in DFT scans, high up on the repulsive wall. This transformation maps ρ ∈ [0, 1] for all separations R which simplifies and stabilizes the fitting.47,48 For an analytical representation of the interaction energies, the following parametrization is used:

\[ V(R, \rho, \theta) = \sum_{\lambda=0}^{10} V_{\lambda}(R, \rho) P_{\lambda}(\cos(\theta)), \]

where \( P_{\lambda} \) are Legendre polynomials and the \( V_{\lambda} \)s are double Morse-like potentials

\[ V_{\lambda}(R, \rho) = D_{1,\lambda}(R) \cdot (1 - e^{-\beta_{1,\lambda}(R)(\rho - \rho_{1,\lambda}(R))})^2 + D_{2,\lambda}(R) (1 - e^{-\beta_{2,\lambda}(R)(\rho - \rho_{2,\lambda}(R))})^2 - \alpha(R). \]

Each parameter \( X_{\lambda} \), including \( D_{1}(R), \beta_{1}(R), \) and \( \rho_{1}(R) \), is fitted to an expression \( X_{1}(R) = \frac{1}{2} p_{1} \tanh(p_{1}(R - p_{2})) + p_{3} \). This parametrization scheme was already found useful for investigating proton transfer between a donor and an acceptor and allows to work with analytical derivatives required for MD simulations.47,48

The parameters \( p \) were determined from least-squares fitting by using a Nelder-Mead simplex\(^{49,50}\) followed by a nonlinear least squares refinement with the I-Noll software.51,52

For the unligated state, the dissociated CO molecule was described by a three-point fluctuating charge model\(^{55}\)

\[ q_{i}(r_{\text{CO}}) = A_{i} + B_{i} \cdot r_{\text{CO}} + C_{i} \cdot r_{\text{CO}}^2 + D_{i} \cdot r_{\text{CO}}^3, \]

where the \( q_{i} \) are the charges on the C and O atoms, respectively (see Table I), and an additional charge was placed at the COM of CO with

\[ q_{\text{COM}} = q_{C} + q_{O}. \]

Such a model was preferred in the present case over a more rigorous multipolar model for computational efficiency.54 The C–O bond potential is an anharmonic and spectroscopically accurate rotational Rydberg-Klein-Rees (RRKR) potential.55–57 This model has performed well in previous studies.53,58

### C. Photodissociation and ligand transfer

Photodissociation was simulated based on the sudden approximation.59 Starting from the bound state (FeCO), the ground state PES was instantaneously switched to the parameters of the unbound state and a repulsive term between the Fe and the CCO atoms was added

\[ V_{\text{repulsive}}(r) = C \cdot \left( \frac{r}{\sigma} \right)^{-12}, \]

where σ is the equilibrium distance of the Fe–C bond (σ = 1.9 Å) and C = 10 kcal/mol is an energy parameter. This potential mimics the repulsive part of the excited state PES to which the Fe–C bond is pumped by the photodissociating laser pulse.4,60 We note that such a preparation of the system does not necessarily redistribute the excess energy correctly into the available degrees of freedom of the leaving CO-fragment. However, as we are primarily interested in preparing a representative ensemble of photodissociated initial structures and because the dissociative potential is typically only active for a few vibrational periods (a few tenths of femtoseconds) in the present case, a more rigorous treatment was not deemed necessary. More appropriate treatments of such processes have been recently discussed in the context of photodissociation of small molecules together with final state analysis.61 After applying the photodissociating pulse, the system is switched back – after time delays ranging from 20 to 200 fs – to the ground state surface in order to follow the reactive dynamics. For this the bond between Fe and CO was removed and replaced by Eq. (6) which was active for 100 fs after which the Fe–CO nonbonded interactions were reintroduced. This included in particular the van der Waals and electrostatic interactions between the CO ligand and its environment. For the electrostatic part, the fluctuating three-point charge model (see Sec. II) was employed. The fitted, three-dimensional PES is reactive and allows to describe both, the Fe–CO and Cu–CO states. However, alternative procedures to follow chemical reactions in condensed-phase simulations are available, including reactive molecular dynamics,62–65 reformulations of it,66 or the empirical valence bond (EBV) formalism.55

### III. RESULTS AND DISCUSSION

#### A. PES fitting

The PES was parametrized by fitting to DFT-reference energies. Figure 2 shows a model of the active site that was used for generating the three-dimensional PES corresponding to the singlet ground state. The model includes the truncated Heme \( \alpha_{1} \) in which the isoprenoid chain was replaced by a methyl group, the CO ligand, the Cu\(^{2+} \) cation, and the His276, His325, His326, and His411 side chains which were truncated to the methyl-imidazole core. In order to estimate how strongly the structure deforms when CO is bound to either Cu\(_{B} \) or the Heme \( \alpha_{1} \), partial optimizations were carried out with the positions of the His-C\(_{B} \) carbon and the carbon atoms of the propionate-carboxylic groups being fixed (see Figure 2). As it is expected the CO–Cu\(_{B} \) bound structure (θ = 180°) is \( \approx \)16 kcal/mol higher in energy than the CO-heme \( \alpha_{1} \) one

<table>
<thead>
<tr>
<th>Atoms</th>
<th>A (e)</th>
<th>B (eÅ)</th>
<th>C (eÅ(^2))</th>
<th>D (eÅ(^3))</th>
</tr>
</thead>
<tbody>
<tr>
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<td>−11.011</td>
<td>20.205</td>
<td>−13.149</td>
<td>3.0461</td>
</tr>
<tr>
<td>O</td>
<td>−11.254</td>
<td>22.309</td>
<td>−15.981</td>
<td>3.9834</td>
</tr>
</tbody>
</table>
FIG. 2. Optimized structure of the active site at the B3LYP/6-31G(d,p) level for CO bound to heme-Fe. The histidine Cβ carbons and the carbon atoms of the propionate-carboxylic groups, marked blue, are fixed at their X-ray positions in the partial optimizations. ($\theta = 0$). In the partially optimized structure, the most significant structural differences are found for the Cu$_B$ atom (its displacement relative to the reference structure is 0.38 Å) and the heme-a$_3$-Fe (displacement 0.15 Å). However, the overall structure is well preserved which establishes that using the X-ray structure is a meaningful reference for scanning the PES.

The minimum barrier of the transfer reaction on the ground state PES is 38.1 kcal/mol and the root mean square error between the parametrized PES and the DFT reference energies is 1.2 kcal/mol. A direct comparison between the reference data and the fit is reported in Figure 3(c) for the cut with an Fe–Cu distance of 5.2 Å and demonstrates the high quality of the PES.

B. Dynamics of CO bound to heme a$_3$ Fe

As a first validation of the parametrized PES, $NVT$ simulations were carried out for Fe-bound CO. The average Fe–C bond length is 1.93 ± 0.067 Å, the average Fe–C–O valence angle is 176 ± 2.04° – close to linearity – and the average Cu$_B$–O distance is 2.39 ± 0.538 Å. The structural data reported for the bovine heart CC$^5$ and the one of Th. thermophilus$^{68}$ are summarized in Table II. Significant differences are, however, observed for the CO-tilt (Fe–C–O angle). The canonical Fe–CO conformation is linear$^{18}$ which is also found in the present MD simulations but is very different in Th. thermophilus. Part of the difference could be related to the rather low resolution of the X-ray diffraction experiments of 2.8–2.9 Å and 2.8–3.2 Å and the low temperature of 100 K at which these experiments were carried out.$^{5,68}$ The tilt observed in the X-ray structures has been proposed to result from “repulsion between the O-atom of the Fe-bound CO and Cu$_B$.”$^{68}$ However, given the experimental data on related systems and the fact that the partial optimizations at the B3LYP/6-31G(d,p) level (see above) based on the X-ray structure yield an almost linear Fe–CO arrangement with an angle of 178°, this appears to be unlikely. Furthermore, a more recent structure for CcO from Bos taurus at 2.2 Å resolution shows an Fe–C–O angle of 164°, considerably closer to the computed value (see Table II).$^{69}$ The current results are also supported by previous computational studies of heme-CO systems.$^{11,53,59,70,71}$ Even at the mixed QM/MM level for MbCO in which the His-Heme-CO system was treated quantum mechanically and the rest of the protein was represented as point charges, a linear Fe–CO arrangement was found.$^{31}$

The Fe–Cu distance during the equilibrium dynamics is 4.98 ± 0.08 Å (see Figure 8). This compares with an average distance of 4.4 ± 0.34 Å from simulations for the unligated protein (see below) and 4.5 Å from the X-ray structure.$^{23}$ Hence, the presence of the ligand in the active site pushes the two metal centers apart and photodissociation of the ligand allows the Fe- and Cu-atoms to move closer. Also, the comparison between simulations and experiment for the
unligated system are an additional validation for the force field employed in the present work.

C. Free CO dynamics

In order to better characterize the active site, simulations were also carried out for photodissociated CO in the bimetallic active site. For this, a quadrupolar electrostatic model for the free CO molecule was employed. This model describes the interaction of CO with the protein better than a conventional point charge model. In particular, the molecular dipole and quadrupole moments are correctly captured and thus fluctuate with CO bond length. This also allows to determine the CO-infrared spectrum from the time-varying dipole-moment autocorrelation function $C(t) = \langle \mu(t)\mu(0) \rangle$.

The total simulation time for free CO in the bimetallic site was 11 ns. This allowed to map out the shape and the effective volume of the cavity around the active site to which free CO has access, as reported in Figure 4. In the free dynamics simulations, we find three possible locations to which CO escapes after photodissociation: the distal pocket, the water-protein interface, and the “K-path” shown in Figure 4(b). Most of the time CO remains in the distal pocket. In one simulation, CO moved towards the protein-water interface and in one it diffused into the “K-path” pocket. In cases where CO left the distal pocket, the escape occurred on a timescale of $\approx 0.5$ ns.

During these simulations the heme $\alpha_3$ Fe changed from a hexacoordinated to a pentacoordinated state. Hence, the Fe moves below the heme $\alpha_3$-plane. This coordination change can be described by the distance between the plane defined by the four heme-nitrogen atoms and the Fe atom. In the original crystal structure, the heme-Fe separation is $\approx 0.42$ Å below the plane (in the direction away from the distal pocket) which compares with 0.50 Å from the present simulations (Fig. 5) and 0.35 Å from previous work on myoglobin. After excitation, CO moves in the direction normal to the heme plane. Hence, the CO dynamics right after photodissociation is rather translational than rotational. After 100–250 fs the CO hits the protein and its dynamics is affected by the structure of the pocket of the active site.

As the major function of CcOs is proton transfer, the proton channels such as the K-path often contain cavities sufficiently large to accept water molecules. The K-path is a proton uptake pathway, in which protons are transferred towards heme $\alpha_3$ and heme $\alpha$ sites, and therefore essential.
FIG. 5. Out of plane motion of Fe in heme a$_3$ during free CO dynamics. $\mu$ – expectation, $\sigma$ – standard deviation.

for the function of the protein. In crystal structures of CcOs of other bacteria, e.g., *Th. thermophilus*, up to 3 water molecules can be found in the area close to the “K-path” pocket. Hence, this pocket may be similar in character to the well-known Xe-binding sites in CcO or myoglobin which are also accessible to external ligands, including CO and NO. It was therefore of particular interest to investigate the dynamics of the free CO molecule inside the active site of CcO, as experiments do not give definitive answers about the existence of free CO after photodissociation.

Infrared spectroscopy is suitable to address this point but may lack the sensitivity and resolution for unambiguous detection. For a computational characterization, ten individual 500 ps simulations of photodissociated CO in the active site were run. This time scale is appropriate to obtain sufficiently resolved and converged spectra as recent work on NO in Mb has shown. The simulations were started from Fe-bound CO, the repulsive potential to induce photodissociation was active for 100 fs after which the system was switched to the $^3$A state. The average IR spectrum of the photodissociated, free CO molecule was calculated with a resolution better than 1 cm$^{-1}$ and is reported in Figure 6. For the IR spectra, the real-time dipole-dipole autocorrelation function $C(t) = \langle \mu(t)\mu(0) \rangle$ is calculated and its Fourier transform $C(\omega)$ yields the IR spectrum according to

$$A(\omega) \propto \omega(1 - \exp(-\hbar\omega/(k_BT)))C(\omega),$$

where $k_B$ is the Boltzmann constant, $\hbar$ is the Planck constant, and $T$ is the temperature. The individual spectra differ as they reflect different environments sampled by the ligand but all of them are broad and centered around 2180 cm$^{-1}$ which is the value for a gas-phase simulation of isolated CO. The difference to the 2143 cm$^{-1}$ band known experimentally originates from the finite step size used in the MD simulations and the classical treatment of the anharmonic oscillator. Consequently, an insignificant shift for photodissociated CO in the active site of CcO is predicted relative to gas-phase CO which contrasts with the situation encountered for free CO in the active sites of Myoglobin or Neuroglobin. Figure 6 reports three individual spectra (colored traces) together with the average over all 10 trajectories (black). Of the 10 trajectories, 9 show a largely unshifted spectrum (similar to the red and green traces in Figure 6) relative to gas-phase CO whereas one trajectory (blue trace) has a spectrum shifted to the red. However, even detailed analysis of the atomic motions did not reveal any substantial differences between the configurations that lead to the blue and red/green spectral features. This is shown in Figure 3 in the supplementary material. Hence, the current simulations do not support the notion of pocket- specific spectra such as those found for diatomic ligands in Mb or Ngb.

As anticipated from previous experiments, the expected spectrum of photodissociated CO in the active site is broad which is consistent for the dynamics of a free diatomic molecule in a highly inhomogeneous electric field and a largely open chemical environment: the pocket of the active site is formed by both polar (the tri-histidine (His276, His325, His326) coordinated Cu$_B$, a Tyr$_{280}$ with a hydrogen bond to the hydroxyl of the heme a$_3$) and apolar groups (Val$_{279}$, Ile$_{315}$, Ile$_{347}$). The pocket volume was analyzed using SURFNET. This program finds cavities by covering space lined by a given collection of residues using spheres of different radii ($R_{\min} = 1.0\text{Å}, R_{\max} = 3.0\text{ Å}$), which define the volume of the pocket. For estimating the pocket volume relevant in the present work, 400 frames along a trajectory with photodissociated CO were analyzed. For this, the CO molecule was removed from the structure. The average size of the pocket is 105 ± 32 Å$^3$, which is approximately three times larger than the cavity of the distal pocket in myoglobin. This, together with the low signal-to-noise ratio in the experiments could partially explain the absence of the free CO signal in the experiments.

D. CO transfer following excitation

In a next step, the ligand transfer reaction was investigated. To this end, 1000 independent excitation trajectories for each excitation time $\tau_e$ were run and analyzed. Initially, the excitation was simulated by a repulsive term (Eq. (6)) which was already used in the free dynamics. At defined times after the excitation – including $\tau_e = 20, 30, 50, 100, 150$, and $200$ fs (for each initial configuration) – the interactions were switched to the interaction potential Eq. (2) describing the ground state PES supporting both, Fe–CO and Cu–CO-bound states. For the heme a$_3$, separate sets of simulations were carried out with the pentaheme and the hexacoordinated force fields. In the following, only the results with the pentacoordinated force field are described and comparisons with results from simulations with the hexacoordinated force field are made where it is appropriate. The supplementary material reports the results from simulations with the hexacoordinated force field.

The analysis of excitation trajectories shows that the probability for ligand transfer to yield Cu–CO is only insignificantly affected by increasing the quench delay time (see Figure 7). For all times $\tau_e$, the final state population is prominently peaked at the Cu–CO state which suggests that
following photodissociation from the heme-Fe the CO ligand is efficiently transferred to the copper atom. After photodissociation, the Fe atom moves out of the heme plane defined by the four heme nitrogens to a distance of $\approx 0.5$ Å below the plane within the first $\approx 100$ fs of the simulation. This has also been found in previous work on Myoglobin$^{59,83}$ and the time for breaking the Fe–C bond has been estimated to be on the order of 100 fs.$^{84}$ The Fe-out-of-plane displacement is close to that in the experimental 1AR1 X-ray structure for pentacoordinated heme $a_3$, where it is 0.42 Å.

In equilibrium simulations for Fe-bound CO, the Fe–Cu distance is typically around 5.0 Å (see Figure 8) which increases to 5.5 Å during the transfer within a picosecond. This displacement is caused partially by the out-of-plane motion of the heme Fe. Therefore, the transfer leads to moderate but functionally important changes to the structure of the active site. In particular, the pentacoordinated heme-Fe is “hidden” below the plane which makes it less available for reactions with external ligands. This is in contrast to the tetrahedrally coordinated Cu-ion which always presents an available ligand-binding site to free CO.

The analysis of the trajectories suggests that $\theta$ is a meaningful reaction coordinate to describe the transition between the Fe–CO ($\theta \approx 0$) and Cu–CO ($\theta \approx 150^\circ$) states (as $\theta$ is the angle between heme $a_3$, the center of the CO and the C of the CO, the minimum corresponding to the Cu–CO has a
value of $150^\circ$ in the coordinate system used). The time series $\theta(t)$ obtained from the trajectories (Figure 9) show that CO arrives at the Cu$_B$ site typically within 1 ps. The transfer itself consists of two phases – a ballistic and a diffusive one (Figure 10).

The kinetic curves show that there is a lag time for the transfer reaction, which corresponds to the minimal time required for the CO molecule to move towards the Cu$_B$ site, followed by the rotation (Figure 10). In the simulations, this lag time is the sum of two components: the quench delay time after which the dynamics is driven by the repulsive potential (Eq. (6)) and the true ballistic time, when the potential describing the ground PES is active (Eq. (2)). The estimated lag time is not greater than 300 fs and the true ballistic time is $\approx 100$ fs (Figure S6 in the supplementary material).

To give a quantitative interpretation of the ligand transfer reaction, we further analyze the reactive trajectories. Similar to proton transfer reactions, it is useful to consider geometrical criteria with which to distinguish educt from product states and to subsequently determine the sensitivity of the conclusions on the specific criteria chosen. The geometrical criterion for ligand transfer is $\rho \geq 0.64$ (CO is closer to Cu$_B$ site) and $\theta \geq 150^\circ$, which are the average values of the corresponding variables obtained from the final state analysis ($\rho_{\text{Cu}B-\text{CO}} \geq 0.64 \pm 0.05$, $\theta_{\text{Cu}B-\text{CO}} = 150 \pm 5^\circ$). Hence, if a trajectory matches this criterion, the transfer is considered to be complete. In the following, the process of CO transfer is treated as a first-order chemical reaction. For determining the rate of conversion from Fe–CO to Cu–CO, we use the conversion fraction (Φ) as a concentration term where Φ is the fraction of the trajectories for which CO is transferred to the Cu$_B$ site at a particular time. Fitting the data (see insets in Figure 9) to a first order kinetics yields an estimated rate constant of $3.83 \pm 0.7$ ps$^{-1}$ and a characteristic time for the conversion to Cu–CO of $\approx 260$ fs, which is somewhat more rapid than the value (450 fs) obtained from experiment which is based on an exponential fit of the Cu–CO (2065 cm$^{-1}$) differential transmission spectrum. To further quantify convergence of these results, bootstrapping was used.

For this, only half of the data was randomly selected to compute the observable and an associated error. This was repeated for 250 samples and yields a rate constant of $3.73 \pm 0.8$ ps$^{-1}$, in quite good agreement with the estimated rate constant from using all the data.

For the hexacoordinated state in which the heme-Fe remains closer to the heme plane, the Fe is still available for rebinding to the CO molecule. Hence, at very short quench delay times ($\tau_e \leq 100$ fs) the probability of rebinding to the heme $a_3$-Fe is high (see Figures S2–S5 in the supplementary material). The probability for ligand transfer to form Cu–CO increases with quench delay time and reaches a maximum of close to 100% (see Figure S3 in the supplementary material) for $\tau_e > 100$ fs and does not significantly change even up to 1 ps. After photodissociation, the Fe atom slightly moves out of the heme plane in the similar fashion as in the pentacoordinated state but only to a distance of $\approx 0.10$ Å below the heme plane within the first 20–100 fs of the simulation. Although the equilibrium structures of the penta- and hexacoordinated heme-group are quite different, the rates of conversion from Fe–CO to Cu–CO are similar. The characteristic time increases from 260 fs to 300 fs when simulations are carried out with the hexacoordinated heme force field. Hence, the conclusions about the time scale and nature of the rebinding reactions are robust with respect to force field parametrizations which characterize two extremes (penta- and hexacoordinated heme-Fe) of the physically relevant situation.

**E. Validation of the CO transfer protocol**

The approach used so far assumes a certain timescale $\tau_e$ for the excited state dynamics. Beyond 100–200 fs – depending on the coordination of the heme-Fe – the final state was...
FIG. 9. CO transfer kinetics for different quench time delays – see labels in panels from analysis of 1000 trajectories. Main panels: black points are averages $\langle \theta \rangle$; grey overlapping lines are the individual trajectories, red dashed line shows the point where the quench has been performed. Insets: $\Phi_{Cu-CO}$ is the conversion fraction to the Cu–CO state. At $t = 0$, $\Phi_{Cu-CO}(t = 0) = 0$. The slope of $\ln(1 - \Phi)$ is the rate of forming Cu–CO, see text.

FIG. 10. 40 different reactive trajectories which lead to transfer for different quench time delays. The PES for a specific Fe–Cu separation of 5.2 Å is also reported for reference. Red lines – dynamics on the excited state surface (CO unbound), grey – dynamics on the ground state surface (CO bound to Cu). Green and orange areas show the approximate contour of the termination criteria used to validate the approach for Fe–CO and Cu–CO states correspondingly, so that whenever the CO molecule is found within the area the state is considered to be defined.

found to be insensitive to the particular time spent in the unbound state. In order to independently validate this, additional simulations in the unbound state were carried out. Specifically, 600 trajectories, each with a maximum simulation time of 100 ps in length, were started from the bound state. The Fe–CO bond was broken and the repulsive potential was active for $\tau_e = 50, 100, \text{ and } 200 \text{ fs.}$ Subsequently, the dynamics was continued with the CHARMM standard force field for a pentacoordinated heme $a_3$ and the CO was treated with the 3-point fluctuating charge model, analogous to the free CO simulations, i.e., without the explicit potential $V(R, \rho, \theta)$ (see Eq. (2)).

The time of arrival at the Cu$_B$ site or at the heme $a_3$ was again determined when certain geometrical criteria were met. They included a metal–C$_{CO}$ distance of less than 2.45 Å and a metal–C–O angle greater than 100°. For comparison, the equilibrium Fe–C separation from the X-ray structure is 1.9 Å and the Cu–C distance from DFT calculations is 1.87 Å. With these geometrical criteria, within 1.5 ps after photodissociation 85% (vs. 90% in the hexacoordinated state) of the simulations have a defined state which is either Cu$_B$–CO or Fe$_{a_3}$–CO. In the remaining cases, CO is still unbound and samples the active site regions characterized earlier, see
Figure 4. Specifically, within 1.5 ps of breaking the Fe–C bond in 512 (85.3%) simulations (out of a total of 600) the CO is bound to Cuβ, in 10 cases (1.7%) it rebinds to the Fea, and for 78 (13%) simulations CO remains in a photodissociated state. The kinetics analysis for the validation simulations based on the first order kinetics along the same lines as for the reaction using the explicit reactive 3D-PEs (see above) yield rate constants of 2.54 ps⁻¹ (characteristic time 393 fs) and 3.11 ps⁻¹ (characteristic time 321 fs) for penta- and hexacoordinated heme-Fe, respectively. Within the limitations of the validation simulations this compares favorably with the simulations on the reactive surface.

Similar to the case when simulations were run on the three-dimensional PES suitable to explicitly describe both bound states, Fe–CO and Cu–CO, additional simulations were carried out in which the heme was treated with a hexacoordinate force field. Within the first 1.5 ps after the excitation 90% of the trajectories terminate in a metal-bound state (Figure S5 in the supplementary material). The probability of rebinding to the Fea is higher in this case as the iron atom is readily accessible to the CO ligand due to the smaller out-of-plane distortion (0.1 Å vs. 0.5 Å). Hence, after 1.5 ps 484 (81%) of the simulations find the Cu–CO state, 59 (10%) terminate in Fe–CO state, and in 57 (9%) case CO remains in a photodissociated state or rebind the metal sites on the longer timescale.

IV. CONCLUSIONS

In the present work, the dynamics of CO between the heme a3 and the Cuβ binding sites in CcO were studied based on molecular dynamics simulations using an accurate three-dimensional reactive PES. Independent on the details of the simulation protocols, the time scale for ligand transfer after photodissociation from the heme-Fe is on the sub-picosecond time scale. Following photodissociation from the heme a3-Fe, approximately 90% of the ligands rebind on such time scales to either Cuβ (majority) or the heme a3 (minority). The data obtained from MD simulations and experimental data suggest a ballistic contribution to the transfer process. A time delay of ≈100 fs before re-binding starts is found in the simulations. This qualitatively agrees with experiment where the time scales were determined by analyzing spectroscopic signatures. The characteristic time scale for rebinding is ≈300 fs compared with 450 fs from experiment. The remaining population (10%) consists of unbound CO sampling the active site which, however, are difficult to directly observe experimentally. Computational infrared spectroscopy suggests that the absorption features of unbound CO are broad and centered around the gas-phase value. All these observations agree with experiments which were interpreted based on spectroscopic data.

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