CO migration pathways in cytochrome P450_{cam} studied by molecular dynamics simulations

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Abstract

Previous laser flash photolysis investigations between 100 and 300 K have shown that the kinetics of CO rebinding with cytochrome P450_{cam}(camphor) consist of up to four different processes revealing a complex internal dynamics after ligand dissociation. In the present work, molecular dynamics simulations were undertaken on the ternary complex P450_{cam}(cam)(CO) to explore the CO migration pathways, monitor the internal cavities of the protein, and localize the CO docking sites. One trajectory of 1 nsec with the protein in a water box and 36 trajectories of 1 nsec in the vacuum were calculated. In each trajectory, the protein contained only one CO ligand on which no constraints were applied. The simulations were performed at 200, 300, and 320 K. The results indicate the presence of seven CO docking sites, mainly hydrophobic, located in the same moiety of the protein. Two of them coincide with xenon binding sites identified by crystallography. The protein matrix exhibits eight persistent internal cavities, four of which corresponding to the ligand docking sites. In addition, it was observed that water molecules entering the protein were mainly attracted into the polar pockets, far away from the CO docking sites. Finally, the identified CO migration pathways provide a consistent interpretation of the experimental rebinding kinetics.

Keywords: internal cavities; ligand migration; molecular dynamics; docking sites; ligand rebinding kinetics

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Ligand binding with hemoproteins has been investigated for more than 30 years (Austin et al. 1975). The picture emerging today is that the reaction dynamics are dominated by protein conformational relaxation and ligand migration among protein cavities (Tetreau and Lavalette 2005). Experimental and theoretical investigations have emphasized three functional roles of internal cavities (Brunori and Gibson 2001): (1) They define preferred migration pathways for ligands; (2) they open transient

channels allowing ligand diffusion from the solvent toward otherwise inaccessible buried active sites; and (3) they cause delayed (re)binding by providing transient docking sites for the ligand. Ligand trapping inside internal cavities has been documented by time-resolved crystallography (Srajer et al. 2001; Bourgeois et al. 2003; Schotte et al. 2003; Hummer et al. 2004), cryocrystallography (Brunori et al. 2000; Chu et al. 2000; Ostermann et al. 2000; Schlichting 2000), spectroscopy (Engler et al. 2000; Lamb et al. 2002; Nienhaus et al. 2002; Kriegl et al. 2003), kinetic competition experiments with xenon (Scott and Gibson 1997; Scott et al. 2001; Nienhaus et al. 2003a,b; Tetreau et al. 2004, 2005), and molecular dynamics (MD) simulations (Elber and Karplus 1990; Carlson et al. 1996; Meller and Elber 1998; Amara et al. 2001; Mouawad et al. 2005).

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These studies were essentially concerned with CO migration in oxygen storage hemoproteins, mainly myoglobin, but also other globins such as cytoglobin (de Sanctis et al. 2004a, b) and neuroglobin (Ascenzi et al. 2004). During the last few years we have investigated the reaction dynamics of a series of catalytic hemoproteins: The cytochromes P450 mono-oxygenases. The cytochrome P450 family derives its name from its ability to bind also CO to form a stable but enzymatically inactive ternary complex displaying a strong absorbance at 450 nm. Cytochrome P450_{cam} has become a paradigm for this, functionally more complex, hemoprotein family in which the ligand coexists with the substrate within a well-buried heme pocket. In its reduced Fe^{II} form, this protein catalyzes the stereospecific hydroxylation of camphor (the substrate) at the 5-exo position in *Pseudomonas putida*; camphor is maintained in the distal pocket not far from oxygen (the natural ligand), which is bound to the iron atom of the heme (Poulos et al. 1985, 1987).

Laser flash photolysis investigations of cytochrome P450_{cam}(cam)(CO) revealed a much greater kinetic complexity than for oxygen storage proteins (Tetreau et al. 2005). Four different geminate processes, i.e., recombination of CO from inside the protein, each of them depending on temperature, were taking place between 77 and 300 K. Near 200 K all processes occurred simultaneously as shown by the kinetic rate spectrum of Figure 1. The two faster processes (G^I and G^I_R bands) were attributed to CO rebinding from its primary docking site above the heme, respectively before and after the displacement of camphor closer to the iron atom; this motion of the substrate corresponded to a conformational relaxation of the heme pocket triggered by the removal of CO from the heme. Kinetic competition experiments with xenon indicated that the two slower recombination processes (G_{Mf} and G_{Ms} bands) were due to delayed rebinding of CO subsequent to its migration in secondary

docking sites located in internal cavities of the protein matrix. A crystallographic study of Fe^{III} P450_{cam} under Xe pressure had revealed the presence of four Xe sites (Wade et al. 2004) which could constitute potential candidates for CO docking after photodissociation. However, in the absence of crystallographic data on trapped CO intermediates or of time-resolved crystallography experiments, the actual ligand docking sites and migration pathways remain undetermined.

To provide a structural and dynamic interpretation of the rebinding processes, we have performed MD simulations of the cytochrome Fe^{II} P450_{cam}(cam)(CO) ternary complex at three different temperatures, 200, 300, and 320 K. These temperatures were chosen in order to explore the ligand migration by favoring either its confinement or its escape from the protein. The results suggest that some sites are more readily accessible to CO and that two of them correspond to identified Xe binding sites. The simulations also reveal that the docking sites correspond to internal cavities, most of which existing independently of the presence of CO.

Materials and Methods

All calculations started using the coordinates of the Fe^{II} P450_{cam}(cam)(CO) complex taken from the Protein Data Bank (PDB entry 3CPP; Raag and Poulos 1989). The polypeptide chain, consisting of 405 residues (residue 10–414), did not contain coordinates for the first nine amino acids. An acetyl group was added to residue 10 of the N-terminal part to avoid the artificial introduction of a positive charge in this position. The tautomeric forms of the histidines were assigned in a way that favored the formation of hydrogen bonds. To simulate photodissociation of the CO complex, the Fe–CO bond of the starting structure was cut before any calculations. We included all the internal crystallographic water molecules that were located within a distance of 16.5 Å from the center of mass of the protein along its first and second inertia axes and of 9 Å along the third one. This resulted in a total of 21 water molecules, to which the TIP3P model was applied (Jorgensen et al. 1983).

The force field parameters

All simulations, including those in the water box, were performed using the parameter set QUANTA/CHARMM20 (Accelrys), in which only polar hydrogens were considered explicitly for the polypeptide chain and camphor, whereas all hydrogen atoms were introduced for the heme group (taken from CHARMM22; MacKerell et al. 1998). For camphor the parameters were obtained from the XPLO-2D program (Kleywegt and Jones 1997) and the partial charges of camphor were taken from Helms and Wade (1997). The simulations were performed using the CHARMM program (Brooks et al. 1983).

The procedures

Simulations with the protein in the vacuum

The same procedure was followed for the various trajectories in the vacuum. The structure was first subjected to a slight

Figure 1. Normalized rate spectrum describing the kinetics of CO rebinding with cytochrome P450_{cam}(cam) at 200 K, obtained by the “Maximum Entropy Method” (Tetreau et al. 2005). $P(\log k)$ represents the probability of CO rebinding with a rate constant equal to k (s^{-1}). Four different geminate rebinding processes are observed, the fastest being G^I .

energy minimization, during which the conjugate gradient method was used for 600 iterations under mass-weighted harmonic constraints that were applied to the system in order to prevent abrupt deviations from the crystal structure. The harmonic force constant was decreased every 100 steps taking the successive values of 250, 100, 50, 25, 10, and 5 kcal/mol/Å². Finally, the energy was minimized for 10 additional steps without harmonic constraints.

The minimized structure was then heated up to 200, 300, or 320 K, depending on the trajectories, by 25 K increments of temperature every 5000 steps. The SHAKE procedure (Ryckaert et al. 1977) was applied on bonds involving a hydrogen atom and the time step was of 1 fsec.

The system was further equilibrated for 100 psec at constant temperature following the Berendsen procedure (Berendsen et al. 1984) with a time coupling to the heat bath of 0.05 psec. This was followed by 900 psec of productive dynamics during which the system was coupled to the heat bath every 5 psec.

To truncate the electrostatic and van der Waals interactions the switch function (Brooks et al. 1983) was used within a cut-on/cut-off interval. Two different intervals were tested: One trajectory was calculated with cut-on and cut-off values of 11 and 15 Å, respectively, and a set of trajectories with values of 5 and 9 Å, respectively. The dielectric constant was taken equal to the distance separating the charges.

Simulations with the protein in a water box

One trajectory was calculated with the protein embedded in a water box at constant pressure (1 atm) and temperature (300 K), using the Berendsen method with a compressibility of 4.63×10^{-5} atm⁻¹. The particle mesh Ewald method (Essmann et al. 1995) was applied, with the width of the Gaussian distribution $\kappa = 0.34$ and a cubic B-spline (the order of its interpolation was equal to 4). The dielectric constant was equal to 1. The switch function was applied between 8 and 12 Å for van der Waals energy. The periodic boundary conditions were used.

The water box. A water box of $88 \times 87 \times 66$ Å³ was constructed so that its dimensions exceeded those of the protein by 10 Å in the x, y, and z directions. Its energy was minimized by the steepest descent method (SD) for 500 steps; it was heated in 6 psec to 300 K, then equilibrated for 45 psec with a time coupling of 0.5 psec.

The protein in the water box. The energy-minimized cytochrome P450_{cam} with all its crystallographic water molecules was immersed in the center of the equilibrated water box. All water molecules that overlapped either the protein or the crystallographic waters (i.e., with the distance between heavy atoms <2.8 Å) were removed. Nineteen sodium ions were added randomly in the water box far from the protein to neutralize its net charge. The final system consisted of 48,710 atoms, 3973 of which corresponded to the protein itself (including camphor and CO).

The energy of the system was minimized keeping fixed the protein for 500 steps of SD; then, the energy of the whole system including the protein was minimized for an additional 500 SD steps, followed by 10 psec of heating from 95 to 300 K, and equilibration during 140 psec. Finally, a production run of 900 psec was performed.

The total number of simulations

A total of 37 trajectories were calculated: 36 were in the vacuum including only internal crystallographic waters and one in the explicit solvent. The trajectories in the vacuum differ by the temperature and cut-on/cut-off distances.

The trajectories will be referred to as:

- Set I: 300 K, cut-on/cut-off 11/15 Å (1 trajectory)
- Set II: 300 K, cut-on/cut-off 5/9 Å (15 trajectories)
- Set III: 200 K, cut-on/cut-off 5/9 Å (10 trajectories)
- Set IV: 320 K, cut-on/cut-off 5/9 Å (10 trajectories)
- Set V: 300 K, in a water box (1 trajectory).

In each set, the trajectories differ by the initial random assignments of velocities.

Calculations of protein cavities

The internal cavities of cytochrome P450_{cam} were calculated by the program Alpha Shapes (Liang et al. 1998a,b), with a probe size of 1.4 Å. A home-built software was used to visualize them; it consisted of filling the empty space of cavities with hydrogen atoms and drawing their contour surface.

Results

We performed preliminary calculations in order to define which procedure was the most appropriate. For this purpose we calculated trajectories of 1 nsec at 300 K, in the vacuum and in a water box, using two different cutoffs.

Choice of the parameters

All calculations yielded satisfactory results both for fluctuations (Fig. 2) and rms deviations (Fig. 3). This was the case for calculations in the water box (set V) and in the vacuum (sets I and II), even when smaller cut-on/cut-off distances were used (set II).

The quality of the trajectories as well as the location of the cavities and the ligand migration pathways (see below) were similar in trajectories calculated in the water box and in the vacuum (with both cut-off distances). Thus, the strategy adopted was to perform a large number of simulations in the vacuum in order to get a relatively representative ensemble of trajectories within acceptable time limits.

Thirty-five trajectories were calculated at three different temperatures; 200 K (10 trajectories, set III) were used to compare with experiments; 300 K (15 trajectories, set II) and 320 K (10 trajectories, set IV) were chosen to accelerate ligand migration and escape from the protein, because the length of the trajectories (1 nsec) was too short to provide a good description of the experimental events that occur on a time scale between 50 nsec and several seconds.

Simulations with the protein in the vacuum

CO migration pathways

The position of CO along the trajectories was monitored using the distance of its center of mass to the heme iron atom. In all trajectories CO moved from the iron during the heating phase and then either came back over the metal or migrated away toward other regions of the protein.

In the following, we will present only the analysis of the trajectories at 300 K (set II), unless otherwise stated.

Figure 2. Fluctuations of the C α atoms along representative trajectories at 300 K (gray lines) compared to the B-factors taken from the crystal structure (pdb file 3cpp, black lines). Calculations in the vacuum, (A) with cut-off interval 11/15 Å (set I), (B) with cut-off interval 5/9 Å (set II). (C) Simulations in a water box with Ewald summation for electrostatics (set V). In calculations in the vacuum the internal crystallographic water molecules were included.

Figure 4 shows that, in five trajectories (# 3, 5, 6, 8, and 15), CO remained confined above the heme during almost the whole time course and that only in one simulation (#2) did it escape from the protein. In all other cases, CO jumped very rapidly (≈ 1 psec) between different regions, where it remained confined for several picoseconds.

CO docking sites. The ligand diffusion paths in each of the 15 trajectories at 300 K are visualized in Figure 5 together with those at 200 and 320 K. At 300 K, six sites of confinement (S1–S6) were identified, all located on the same moiety of the protein.

The average CO–Fe distances were about 4, 5.5, 9.5, 13.3, 7.1, and 10 Å for S1 to S6, respectively (Table 1). To explore the migration pathways more precisely, the fraction of time spent by CO in the vicinity of each protein residue (i.e., when the distance between the residue's heavy atoms and the CO atoms was <4 Å) was determined. Then the results for all trajectories were

compared, and the maximum percentage of residence time near each residue was retained to display all possible pathways. It is apparent in Figure 6 that CO took preferred directions and spent most of the time in well-defined locations: Indeed, the CO ligand came in the vicinity of only 31 of the 405 residues of P450_{cam}.

This analysis allowed us to define the CO docking sites precisely. The residues lining them are presented in Table 1. Two sites, S1 and S2, are located on the distal side of the heme: S1 above the four heme's nitrogens, between camphor and helix I, and S2 between heme's pyrroles C and D and helix I. Two other sites, S3 and S6, are located on the edge of the heme: S3 between pyrrole D and helices C and I and turn 6, and S6 between pyrrole C and helices D, E, I, and L, near the region occupied by three structural water molecules (W23, 66, and 186) (Raag and Poulos 1989). Site S5 is on the proximal side of the heme, between pyrroles C and D and helices C, I, and L. Finally, one site, S4, is located far from the heme, between helices C, H, and I and turn 9.

Similar sites were observed at 200 and 320 K. However, at 200 K only S1, S2, and S3 were populated; at 320 K, S4 was split in two parts, S5 was more extended, and a nearby site S7 could be distinguished (Fig. 5) between helices E, G, H, and I, at an average distance between the center of mass of CO and Fe of 12.7 Å. Sites S1–S7 are in an essentially non-polar environment, suggesting that they might correspond to hydrophobic cavities; this point will be discussed below.

The percentage of the CO occupancy of the different sites, calculated from the sum of occurrences in all trajectories at each temperature, is given in Table 1. It appears that S2 is depopulated in favor of the other sites upon increasing temperature. Site S7 is populated only at 320 K.

The sites being well defined, the migration pathways of CO versus time can be described from the simulations. The passage from S1 to S3 takes place necessarily via S2,

Figure 3. C α rms deviations from the crystal structure along the same trajectories as in Figure 2: set I (gray line), set II (black line), and set V (dashed line). (Inset) Main features concerning the conditions of the simulations and the cut-on/cut-off values of the switch function.

Figure 4. Distance of the CO center-of-mass to the iron atom vs. time along the 15 trajectories of set II (at 300 K). The docking sites (S1 to S6), in which CO remains confined at a given time, are indicated.

as can be seen in trajectories (Traj.) 1, 7, 9, 10, 12, 13, and 14 of Figure 4; from S3, CO can migrate toward either S4 (Traj. 9) or S5 (Traj. 1, 12, and 13); CO can reach S6 starting from either S1 (Traj. 6), S2 (Traj. 11) or S5 (Traj. 12). Finally, S7 is only populated from S6 (observation made at 320 K, data not shown). The same migration pathways are observed at the three temperatures; they are summarized in Figure 7.

In the trajectory calculated at 300 K with cut-on/cut-off distances of 11/15 Å (set I) CO migrated from S1 to S2, then S3 and S5, showing the presence of similar routes of migration (data not shown).

CO escape trajectories from the protein. CO never left the protein in any of the 1-nsec trajectories at 200 K. At 300 K it escaped only in one of the 15 trajectories, starting from S1, first along the normal to the heme plane, then skirting around camphor and leaving between Ala95 of helix B' and Glu198 of helix G; this path will be denoted p2. At 320 K, CO escaped from the protein in four of the 10 trajectories, by following the same direction in three of them: In Traj. 1, 7, and 8, CO escaped along path p1, i.e., from S5 on the proximal side of the heme between helices C and L (more precisely, in Traj. 1, between Val118 on one side and Leu358 and

Figure 5. Snapshots of CO at time intervals of 100 psec along all trajectories at 200 K (set III), 300 K (set II), and 320 K (set IV). The C α atoms of the polypeptide chain from each frame were superimposed on those of the crystal structure and all views are in the same orientation. The CO molecules are colored differently according to the region into which they are localized (blue: S1, cyan: S2, green: S3, yellow: S4, orange: S5, red: S6, and black: S7); the heme and camphor are in gray and pink, respectively. In the *bottom* panel, the entire protein is shown in the same orientation at 320 K with the CO molecules presented as hard spheres, and the secondary structure elements denoted in blue. The views were obtained using the Insight II program (Accelrys).

Leu362 on the other side, in Traj. 7, between Met121 and His361, and in Traj. 8, between Asp116, Gln117, and His361); in Traj. 5, CO escaped along path p3, i.e., from S3 on the edge of the heme between residues Asp125 of helix C and Ile222 of helix H.

Contrary to CO, the crystallographic water molecules (not shown in Fig. 5 for the sake of clarity) remained always well localized at the three temperatures, indicating

that they have a long (>1 nsec) residence time and are therefore likely to play a structural role.

Internal cavities

To address the question whether the docking sites are coinciding with pre-existing internal cavities, the latter were monitored at 50-psec time intervals along selected trajectories (1, 3, and 10 at 300 K and 1 and 2 at 200 K). This choice was based on the criterion of the site occupation by CO with the aim to investigate whether the ligand was influencing the presence of nearby cavities. At 300 K, in Traj. 3 and 10, CO remained confined mostly in S1 and S3, respectively, while in Traj. 1 it was jumping between S3 and S5. At 200 K, in Traj. 1 and 2, CO remained in S3 and S2, respectively. Moreover, cavities were also calculated along two trajectories at 320 K for comparison. Because the presence of CO prevents a sound evaluation of the size of the cavity into which it resides, the ligand was removed from the frames before performing the cavities calculations, contrary to water molecules which were maintained. In each selected frame of each trajectory, a large number of cavities (>20) were found; they were spread throughout the protein matrix, and their shape, size, and connections changed from frame to frame (Fig. 8). The volume of the largest cavity was >400 Å³. At 300 K, eight cavities (A–H) with a noticeable size (>50 Å³) were found recurrently in many frames from selected trajectories. Because the cavities frequently changed in size and shape, a precise definition cannot be given, but their location can be characterized by the residues that most frequently bordered their walls in the structures sampled along the trajectories. Table 2 shows that all cavities have a large nonpolar surface area. Three of them (D, F, and H; see Fig. 8) are located far from the CO pathways, whereas cavity G, located near camphor, is along one of the CO escape paths (p2) described above. The four remaining cavities, A, B, C, and E, correspond to the CO docking sites: Cavity A to S1 and S2, B to S3 and S5, C to S7, and E to S6. The coincidence between docking sites and internal cavities is clearly apparent in Figure 9A.

Seven cavities (B–H) were observed independently of the CO position; in contrast, cavity A, located in the central distal pocket, shifts when CO moves from S1 to S2 and disappears when CO migrates to other sites because of the correlated motion of camphor, which then occupies the cavity position (Fig. 10). Finally, the location of the eight cavities was independent of the temperature, although their sizes were generally smaller at 200 K and larger at 320 K.

Simulations with the protein in a water box

One trajectory was calculated with the protein fully solvated into a box of 14,906 water molecules. An

Table 1. Environment and characteristics of the seven identified CO docking sites

Site	Fe–CO ^a distance	Environment	Observed occupancy ^b (%)		
			at 200 K (<i>n</i> = 10)	at 300 K (<i>n</i> = 15)	at 320 K (<i>n</i> = 10)
S1	4.0	heme (nitrogens), cam helix I: L244, V247, G248, T252	38	36	27
S2	5.5	heme (pyrroles C and D), cam helix I: L244, L245, G248, G249	33	7	3
S3	9.5	heme (pyrrole D) turn 6: P100 helix C: F111, R112, A115 helix I: M241	29	27	45
S4	13.3	helix C: F111, L114, A115 helix H: I222, V223 turn 9: V228 helix I: M241	0	7	1
S5	7.1	heme (pyrroles C and D) helix C: A115, V119 helix I: L245 helix L: L358, G359	0	17	14
S6	10.0	heme (pyrrole C) helix D: L127 helix E: F163, L166 helix I: G249, V253 helix L: L362, E366 water: 23, 66	0	6	6
S7	12.7	helix E: F163, A167, L169 helix G: L204 helix H: I220 helix I: C242, L245, L246	0	0	4

n is the number of trajectories over which the percentages were calculated.

^a Average distance between CO center-of-mass and Fe (Å).

^b Relative CO occupancy (in percent) of each site calculated from the sum of occurrences in all trajectories at 200, 300, or 320 K.

important hydration of the protein was observed during the trajectory, the number of internal water molecules increasing from 21 (the crystallographic waters) to 79.

Remarkably, this water flux did not modify the characteristics of the internal cavities which were found again in almost the same locations and with the same sizes as in the vacuum. Once again cavities D, F, and H were located far from the CO pathways, G, along the escape path p2 and A, B, C, and E in the CO docking sites. In fact, water molecules were entering and leaving the protein by following completely different pathways from those described for CO in the vacuum (Fig. 9B). They were mainly attracted into the polar pockets already occupied by crystallographic waters, avoiding the moiety of the protein corresponding to the docking sites S1 to S7 (except S6, which is close to structural water). In this trajectory, it happened that the CO ligand moved only between sites S1 and S2 as it was the case for several trajectories calculated in the vacuum. However, the presence of the internal cavities A, B, C, and E, that include sites S1, S2, S3, S5, S6, and S7, indicates that the CO pathways should not be significantly modified by the presence of water around and inside the protein.

Discussion

Simulations of the protein were performed under various conditions, using different cut-off distances (11/15 Å or 5/9 Å) or procedures (in the vacuum with only internal crystallographic waters or in an explicit water box). In all these simulations, it was observed that the cavities were located in the same regions of P450_{cam} and that the routes of CO migration were similar. The robustness of these results suggests that both were mainly determined by the tertiary structure of the protein.

Internal cavities

Internal protein cavities have been studied both theoretically (Wade et al. 1991; Bakowies and van Gunsteren 2002; Radresa et al. 2002; Teeter 2004; Vaitheeswaran et al. 2004; Rashin and Rashin 2005) and experimentally (Tilton et al. 1984; Prangé et al. 1998; Rubin et al. 2002). An increasing number of crystallographic studies revealed that the cavities may be occupied by xenon atoms when they are hydrophobic or by more or less mobile water molecules in polar environments. A number of theoretical methods have been developed to calculate

Figure 6. Representation of the residues closely approached by CO (at a distance <4 Å) during the time course of the 15 simulations of set II at 300 K. Residue numbers 415 and 417 correspond to heme and camphor, respectively. The height represents the maximum percentage of time that CO spent in the vicinity of each residue of the protein in any trajectory; only percentages exceeding 2% are shown for clarity.

their distribution and localization inside proteins, as well as their hydration. Both crystallographic studies and MD simulations performed on wild-type or mutant myoglobins (Elber and Karplus 1990; Carlson et al. 1996; Hummer et al. 2004; Radding and Phillips 2004; Bossa et al. 2005), hemoglobin (Mouawad et al. 2005), and truncated Hb (Milani et al. 2004) suggested that internal cavities play a functional role and partly determine the protein reactivity by defining preferred diffusion pathways and providing transient docking sites for ligands.

The first argument in favor of the presence of a large number of atom-sized voids in cytochrome P450_{cam} came from Williams et al. (1994). More recently, crystal structure of P450_{cam} in the presence of xenon revealed the presence of four Xe binding sites (Wade et al. 2004). However, this information was not sufficient to identify CO migration pathways and docking sites unambiguously and to correlate them with the observed geminate rebinding processes.

In our simulations, a large number of small cavities were found to appear and disappear dynamically along the trajectories (Fig. 8). Only eight with a noticeable size were persistent; they were in an essentially nonpolar environment (Table 2). We observed that the opening/closure of the passage between adjacent cavities was not strictly correlated with the presence of nearby CO, contrary to what was described previously for myoglobin (Carlson et al. 1996; Bossa et al. 2004). Except for the distal cavity A, which was located above the heme, the seven other cavities were observed independently of the CO position; their formation was rather determined by the structure of the protein. For instance, the largest and most persistent cavities (including those corresponding to docking sites) were observed in trajectory 3 (at 300 K) in which CO remained confined in site S1 above the heme. However, the presence of CO inside a cavity maintained it

open for a longer time although it did not influence the size of adjacent cavities.

Similar cavities were also observed in the simulation of the protein in a water box but amazingly no water molecules entered these cavities (except F) although the protein was highly hydrated during the trajectory. Interestingly, this does not seem to be due to the hydrophobic character of the cavities, since cavity F, which is as hydrophobic as the others, was hydrated. A possible explanation may be based on the observation of Williams et al. (1994), who reported that in α -helical proteins the cavities were less hydrated than in other proteins and attributed this observation to the smaller potential number of hydrogen bonds that water could establish with the protein backbone already involved in intra-helical interactions. In our case, except A and F, all other cavities were mainly located between α -helices. In cavity A, water entry was prevented by the permanent presence of CO in this trajectory, while cavity F, surrounded mainly by turn T2 and one strand of β 5, was filled with water molecules.

CO docking sites and migration pathways

In the present simulations, the diffusion of the CO ligand was free of any bias such as those resulting from artificial restraints or forces, or multicopy sampling. Nevertheless, the ligand did not follow arbitrary paths inside the protein but moved toward preferred regions into which it remained transiently confined. Seven docking sites (S1 to S7) were identified. The residues lining them were mostly hydrophobic (Table 1).

The CO migration consisted in jumps from site to site through well-defined paths. The transitions between adjacent sites were almost instantaneous while CO remained

Figure 7. Schematic representation of the CO docking sites and routes of migration in the same orientation as Figure 5. The slanted letters A, B, C, and D designate the pyrrole rings of the heme. The ellipses represent sites S1 to S7; their sizes are roughly related to the relative CO occupation of the sites. Straight and curved arrows indicate the migration and escape pathways, respectively. The thickness of the arrows reflects the number of passages from site to site observed from all trajectories of sets II, III, and IV. It can be seen that CO migrates the most frequently from S1 to S2 then S3 and finally S5, and that it escapes from S5 along p1.

Figure 8. Fluctuations of cavities along trajectory 3 at 300 K. All cavities taken from only eight frames at 100-psec time intervals are presented. Their names are indicated in the last panel. The views have the same orientation as in Figure 5.

confined for comparatively long times in each site. The transitions were sometimes accompanied by side-chains motions (e.g., Phe111 and Met241 during the passage of CO from S3 to S4), but always by a slight tilt of the adjacent helices. These observations are in good agreement with those reported in other studies of CO migration in Mb (Schotte et al. 2003; Hummer et al. 2004).

In P450_{cam}, not all sites were directly connected with each other; in particular, S4 could be reached only from S3, and S7 only from S6; in contrast, S6 could be accessed from S1, S2, or S5 (Fig. 7).

Ligand escape from the protein

As a rule, entry and escape of ligands are not directly observable in experiments because they do not give rise to any spectroscopic signal. However, escape can be quantified provided it competes with some rebinding process. Experimental evidence for the presence of two escape pathways was reported only recently in Mb, but this case was rather exceptional (Tetreau et al. 2004). MD simulations provide the adequate and complementary alternative to the description of these processes.

In P450_{cam}, CO followed three different pathways to escape from the protein, p1, p2, and p3 (Fig. 7). In p1, the CO located in S5 left the protein between helices C and L, in p2 it escaped from S1 through helices B' and G, and in p3, it left from S3 between helices C and H. Pathways p1 and p2 were comparable to those observed by Lüdemann et al. (2000a,b) for the camphor substrate, using a Random Expulsion Molecular Dynamics method, in which an artificial randomly oriented force was imposed to the substrate. These authors have identified three paths (pw1, pw2, and pw3). The main path for camphor with the lowest energy barrier, pw2, corresponded to the present p2, from which CO escaped rapidly (Traj. 2 in Fig. 4) without exploring docking sites, likely because the channel could widen easily enough to permit the passage of camphor; pw1, the camphor path with the highest energy barriers, corresponded to p1, the passage from which CO escaped most frequently and along which lay the most populated docking sites, S3 and S5. Finally, pw3, located either between helices F and G or near EF loop, was completely different from p3 but was close to the docking site S7. In the present simulations, camphor remained close to its initial position because the trajectories were not long enough to allow spontaneous important displacements of such a large-sized molecule.

Both recent experimental and theoretical studies that have monitored the migration routes of camphor and other large-size substrates in P450_{cam} have shown that the substrate escapes from the protein without exploring docking sites (Lüdemann et al. 2000a, b; Dunn et al. 2001; Pylypenko and Schlichting 2004). We can see here that CO adopts a similar behavior when it leaves the protein along the main substrate path, p2, whereas when following other routes, it spends time in docking sites.

Comparison between CO docking sites, cavities, and xenon binding sites

In previous MD simulations of Mb, it was reported that CO hopped rapidly between pre-existing cavities (Elber and Karplus 1990; Bossa et al. 2005) or that the opening/closure of the passage between adjacent cavities was strictly correlated with the presence of CO nearby (Carlson et al. 1996; Bossa et al. 2004). In cytochrome P450_{cam}, both situations were encountered. Most sites (S2, S3, S5, S6, and S7) corresponded to pre-existing cavities among which CO migrated, whereas in site S1 competition between CO and camphor prevented the formation of a cavity (this point will be discussed below) and site S4 was only formed in the presence of CO nearby.

On the other hand, the four xenon sites that were identified by crystallography (Wade et al. 2004) coincided with some hydrophobic cavities calculated here. Indeed, Xe1 corresponded to both cavity C and site S7,

Table 2. Residues lining the eight biggest (volume >50Å³) persistent cavities (Cav. A to Cav. H) in cytochrome P450_{cam}

Cav. A (S1, S2) ^a	Cav. B (S3, S5) (Xe4) ^b	Cav. C (S7) (Xe1)	Cav. D (Xe2, Xe3)	Cav. E (S6)	Cav. F	Cav. G	Cav. H
I-β3-β5 ^c CAM HEME	T6-C-I HEME	E-G-H-I	I-J-K-L	C-E-L HEME	T2-A-β5	T5-B'-T6-F- G-I-β5 CAM	E-F-I
I/ Leu244 Val247 Gly248 Thr252 β3/ Val295 β5/ Val396 CAM HEME	T6/ Pro100 C/ Arg112 Ala115 Val118 Val119 I/ Met241 Leu245 L/ Leu358 Gly359 HEME	E/ Phe163 Ala167 Leu169 G/ Leu204 Ile208 Arg211 H/ Ile220 I/ Cys242 Leu245 Leu246	I/ Phe256 Leu257 Phe259 Ser260 Met261 Leu264 J/ Leu274 K/ Ala284 Leu288 L/ Leu371	C/ Val119 E/ Pro159 Ile162 Phe163 L/ Leu362 Ala363 Glu366 HEME	T2/ Met28 A/ Val38 Trp42 β5/ Ser393 Ser397	T5-B'/ Phe87 T6/ Phe98 F/ Met184 Thr185 G/ Phe193 I/ Val247 β5/ Ile395 CAM	E/ Phe163 Ala167 Leu169 F/ Ile174 Leu177 I/ Leu250

The cavities were calculated in structures sampled at 50-psec time intervals along two trajectories at 200 and 320 K, and three trajectories at 300 K.

^aCO docking sites located in the cavity.

^bXe docking sites located in the cavity (see the Discussion).

^cSecondary structure elements surrounding the cavity. Capital letters (A–L) refer to helices, T to turns and β to sheets, as they were defined by Raag and Poulos (1989).

and Xe4 to cavity B and site S5; Xe2 and Xe3 were at the same location as cavity D, far from any CO docking site (Fig. 9C). Although cavity D constitutes a Xe site, CO did not pass through it along any trajectory, presumably because this cavity is close to the surface (accessible to Xe from the solvent) but far from the active site (the initial position of CO in our simulations).

Structural interpretation of the CO geminate rebinding dynamics

The present results provide a basis for a structural interpretation of our previous laser flash photolysis kinetic study of CO rebinding with P450_{cam}.

Assignment of the geminate rebinding processes G^I and G^I_R : Heme pocket relaxation

Both the comparison of the crystal structures of the Fe^{II} and Fe^{II}-CO species and energy minimization calculations previously carried out revealed that, upon removal of CO, camphor relaxes toward a position closer by ~0.7 Å to the heme normal passing through Fe. This was attributed to the increase of the steric repulsion between camphor and one heme propionate resulting from the doming of the heme after CO dissociation (Tetreau et al. 2005). These observations led us to propose that the two fastest geminate processes (G^I and G^I_R bands of Fig. 1) were associated with CO rebinding from the primary docking site before and after the substrate relaxation motion, respectively.

This view is further supported by the present MD results. Examination of the CO and camphor distances to the heme normal in all trajectories (Fig. 10) showed that, when CO was at a short distance to the normal (2 Å), i.e., in S1, camphor was pushed away at an average distance of ~4 Å, as in Traj. 8. In contrast, when CO jumped between different sites farther from the heme normal, as in Traj. 12, camphor relaxed to its position in the deligated structure, at a mean distance of 2.6 Å. The latter is in good agreement with the distance observed in the crystal deoxy-structure (2.7 Å) (Poulos et al. 1987). Thus, the G^I and G^I_R bands can be assigned to CO rebinding from primary docking sites S1 and S2, respectively. Although both sites lie above the heme, rebinding must be slower from S2 than from S1 because of the increased steric hindrance of camphor. A further support to this assignment is provided by the temperature dependence of these band amplitudes and of the populations of the sites. Indeed, despite the difference in the temperature range (from 140 to 200 K for experiments and 200 to 320 K for simulations), similar variations were observed with increasing temperature: The intensity of bands G^I and G^I_R decreased significantly, although that of G^I_R more importantly, and, in parallel, the population of the docking sites S1 and S2 decreased but more appreciably for S2 (Table 1).

Assignment of the geminate rebinding processes G_{Mf} and G_{Ms} : Ligand migration

The two other geminate rebinding bands of the rate spectra (G_{Mf} and G_{Ms}) correspond to much slower processes than

Figure 9. (A) Comparison between CO docking sites and internal cavities. The CO molecules taken every 100 psec from all 15 trajectories at 300 K (as in Fig. 5, panel 2, same color code) and all cavities (in gray) located in the same regions, calculated every 50 psec from Traj. 1 (*top*) or Traj. 3 (*bottom*) at the same temperature, are drawn after superposition of the polypeptide chain on that of the minimized structure (only the heme and camphor of this structure are shown). One can see that docking sites coincide with pre-existing internal cavities, except S4. However, cavity A (corresponding to site S1 in blue) is not observed in Traj. 1, contrary to Traj. 3, because in Traj. 1 CO does not reside in site S1, which is therefore occupied by camphor (see Fig. 10). It can be observed in the *top* panel that cavities extending from the *bottom* of site S5 correspond to the CO escape path p1. CO molecules are shown as hard spheres not to scale for clarity. (B) Water penetration in cytochrome P450_{cam} in the simulation in the solvent (set V). A snapshot of the protein corresponding to the last frame of the trajectory is viewed from the distal side of the heme, i.e., rotated 90° from A and C. Only water molecules located inside the protein, within a distance of 16.5 Å from the center of mass of the protein along its first and second inertia axes (represented by the white circle) and of 9 Å along the third one (axis pointing *toward* the reader), are shown. They are colored in blue: dark blue for water that have entered from the bulk, and cyan for crystallographic waters. Helices are in yellow, the rest of the backbone and the heme in light gray, and camphor in dark gray. To visualize the position of the docking sites (S1–S7), CO from all trajectories calculated in the vacuum at 300 K (set II) and 320 K (set IV) have been superposed on the structure and drawn as red hard spheres. It can be seen that water is localized in the less-helical moiety of the protein opposite to the CO docking sites, although in the present trajectory (set V), these sites were free since CO remained confined over the heme (in S1 and S2). (C) Comparison between CO and xenon docking sites. The protein has the same orientation as in A. Xe atoms are shown as red hard spheres (not to scale for more clarity) and CO (taken from all trajectories at 300 and 320 K) are presented as sticks, with the same color code as in Figure 5, except for site S7, which is in white. It is clearly apparent in the figure that Xe1 is near S7 and Xe4 is hidden among the CO molecules that spread from site S5 at 320 K. Xe2 and Xe3 are in cavity D; the residues lining this cavity are drawn as gray sticks.

G^I and G^I_R , suggesting that rebinding is delayed by CO migration to secondary docking sites. This conclusion was supported by xenon competition experiments. At a xenon pressure of 7 atm, the band corresponding to the slowest rebinding process (G_{Ms}) almost entirely disappeared, indicating that the corresponding site was saturated with Xe. In contrast, the faster G_{Mf} process decreased only slightly at higher pressures (Tetreau et al. 2005). These results suggested

that G_{Ms} and G_{Mf} might correspond to rebinding from two different docking sites, the one associated with G_{Ms} having a significantly higher affinity for Xe than the other. Further, these two sites must be occupied either in parallel or sequentially, in which case the site responsible for the slowest rebinding process, G_{Ms} , should be at the end of the sequence.

The analysis of the CO migration pathways from the trajectories (Fig. 7) showed that S3 and S6 are the only

Figure 10. Distance of camphor (gray line) and CO (black line) to the heme normal passing through Fe along two representative trajectories at 300 K (set II): Traj. 8 (top) and Traj. 12 (bottom). It shows that camphor comes close to the heme plane's normal when CO moves away. S1 to S6 indicate the docking sites in which CO is located.

sites directly accessible from the primary docking sites S1 and S2. This makes them good potential candidates for the faster rebinding process (G_{Mf}) especially as their environments are not as hydrophobic as those of other sites. Indeed, the presence of Arg112 in S3 and of Glu366 and two water molecules in S6 could result in relatively low affinity for Xe.

On the other hand, sites S4, S5, and S7, which can only be reached through S3 and S6, should correspond to the slowest rebinding process, especially as they are lined with highly hydrophobic residues, giving them the character of high affinity for Xe. However, in the crystal structure resolved under a xenon pressure of 7 atm, only sites S5 and S7 were occupied by Xe atoms. This may be due to the fact that site S4 does not correspond to a pre-existing cavity and is opened only by the presence of CO. Anyway, the X-ray structure indicates that S4 could not be associated with the G_{Ms} band which disappears at 7 atm of Xe.

This leaves two possible passageways starting from the primary docking sites, either $S3 \rightarrow S5$ or $S6 \rightarrow S7$. A large number of trajectories are needed to obtain converged site occupancies to rigorously discriminate between these two paths. Nonetheless, in the simulations at multiple temperatures S6 and S7 are consistently found to be less populated than S3 and S5. Indeed in Table 1 it can be seen that, while S6 is only populated at $T \geq 300$ K, S3 is already occupied at $T = 200$ K and that, while S7 is filled only at $T = 320$ K, S5 is populated at $T \geq 300$ K. The fact

that S5 is not occupied at 200 K may be due to the short length of the trajectories (1 nsec) compared to the experimental time scale (>50 nsec). From these considerations one can conclude that it is highly probable that the CO migration along the passageway $S3 \rightarrow S5$ is responsible for the experimental observations of the rebinding processes, G_{Mf} and G_{Ms} . Crystallography experiments that would demonstrate the presence of Xe in site S3 at higher pressures or cryocrystallography or time-resolved crystallography visualizing the CO migration pathways after dissociation would be very useful to definitely support these assignments.

At present the most extensive data on CO migration are available for myoglobin and cytochrome P450_{cam}. Except for the heme, these two proteins have structurally little in common. Their identified cavities are not related in any way and they are disposed differently around the heme. However, remarkably, both proteins exhibit a common feature: The presence of a series of cavities connecting the distal pocket to the proximal side of the heme. These connections may be important for a ligand that enters the protein from the proximal side, because it gives it the ability to reach its binding site from this region too. Such a possibility might enhance the ligand binding rate.

Conclusions

In the present simulations, the CO migration pathways in cytochrome P450_{cam} were found to correspond to fast transitions between well-defined docking sites which coincided with pre-existing internal cavities. This emphasizes the functional role of the cavities, although not all of them were visited by the ligand during the 1-nsec trajectories. One of these unvisited cavities (D), however, corresponds to Xe docking sites, suggesting the possible existence of some ligand entries that do not lead directly to the active site. Moreover, the simulations in a water box showed that water molecules did not follow the same paths as CO despite the similar size of the two molecules and the presence of open internal cavities along the CO pathways. This shows that the polarity of the ligand plays a predominant role in its migration within the protein. Finally, the simulations provide a consistent interpretation of the complex kinetics previously reported for CO rebinding in P450_{cam}, not only by confirming the occurrence of heme pocket relaxation and of ligand migration, but also by attributing to each of the four bands observed experimentally a specific location of CO in the protein (S1 to G^I , S2 to G^I_R , S3 to G_{Mf} , and S5 to G_{Ms}).

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