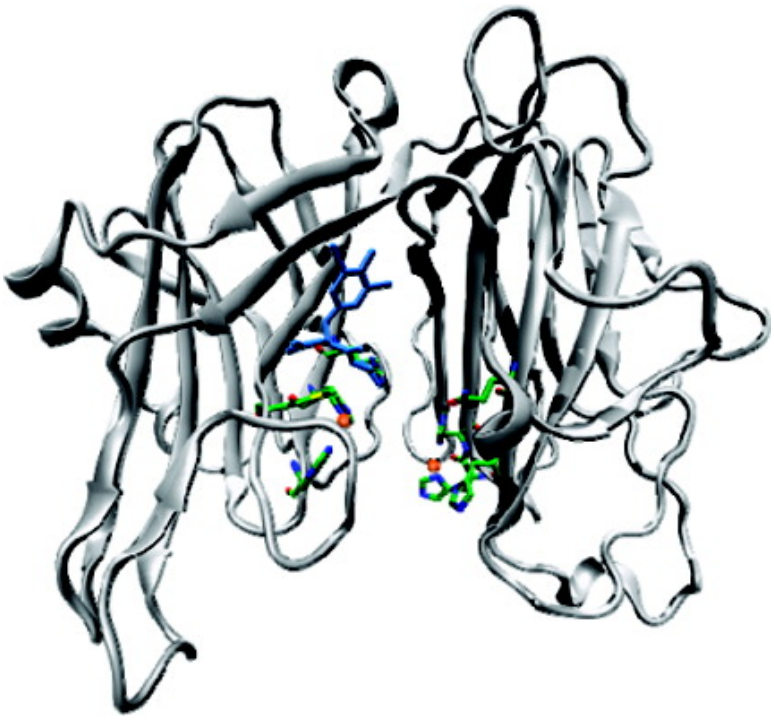
Article

Long Distance Electron-Transfer Mechanism in Peptidylglycine -Hydroxylating Monooxygenase: A Perfect Fitting for a Water Bridge

Aurlien de la Lande, Sergio Mart, Olivier Parisel, and Vicent Moliner

J. Am. Chem. Soc., **2007**, 129 (38), 11700-11707• DOI: 10.1021/ja070329l • Publication Date (Web): 01 September 2007



J|A|C|S
ARTICLES
Published on Web 09/01/2007

Long Distance Electron-Transfer Mechanism in Peptidylglycine α-Hydroxylating Monooxygenase: A Perfect Fitting for a Water Bridge

Aurélien de la Lande,[‡,#] Sergio Martí,[‡] Olivier Parisel,[#] and Vicent Moliner*[,‡]

Contribution from the Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castelló, Spain, and Laboratoire de Chimie Théorique, UMR 7616 CNRS/UPMC, Université Pierre et Marie Curie, Paris 6, Case Courrier 137, 4 place Jussieu, F. 75252 Paris Cedex 05, France

Received January 16, 2007; E-mail: moliner@qfa.uji.es

Abstract: The active sites of copper enzymes have been the subject of many theoretical and experimental investigations from a number of years. Such studies have embraced topics devoted to the modeling of the first coordination sphere at the metallic cations up to the development of biomimetic, or bioinspired, catalytic systems. At least from the theoretical viewpoint, fewer efforts have been dedicated to elucidate how the two copper cations act concertedly in noncoupled dicopper enzymes such as peptidylglycine α-hydroxylating monooxygenase (PHM) and dopamine β-monooxygenase (DβM). In these metalloenzymes, an electronic transfer is assumed between the two distant copper cations (11 Å). Recent experimental results suggest that this transfer occurs through water molecules, a phenomenon which has been theoretically evidenced to be of high efficiency in the case of cytochrome b_5 (*Science*, **2005**, *310*, 1311). In the present contribution dedicated to PHM, we overpass the common theoretical approaches dedicated to the electronic and geometrical structures of sites Cu_M or Cu_H restricted to their first coordination spheres and aim at directly comparing theoretical results to the experimentally measured activity of the PHM enzyme. To achieve this goal, molecular dynamics simulations were performed on wild-type and various mutants of PHM. More precisely, we provide an estimate of the electron-transfer efficiency between the Cu_M and Cu_H sites by means of such molecular dynamics simulations coupled to Marcus theory joined to the Beratan model to approximate the required coupling matrix elements. The theoretical results are compared to the kinetics measurements performed on wild and mutated PHM. The present work, the dynamic aspects of which are essential, accounts for the experimental results issued from mutagenesis. It supports the conclusion that an electronic transfer can occur between two copper(I) sites along a bridge involving a set of hydrogen and chemical bonds. Residue Gln170 is evidenced to be the keystone of this water-mediated pathway.

I. Introduction

In their 2005 paper related to the nature of aqueous pathways within electron-transfer proteins, Lin, Balabin, and Beratan have highlighted the existence of three electronic coupling mechanisms for electronic transfers in various aqueous conditions.[1] Especially, for donor acceptor distances ranging between about 9 and 12 Å, a structured water mediated regime has been evidenced by means of molecular dynamics performed on two interacting cytochrome b_5. For such distances, extended-Hückel electronic coupling calculations show that the electronic transfer is enhanced if paths involving water molecules are taken into account. Moreover, in this case, the decay of the electronic coupling with respect to the donor−acceptor distance exhibits a plateau which reflects a better efficiency of the electronic transfer than in bulk water. This has been rationalized by the emergence of a network of a few water molecules which induces a set of multiple constructively interfering pathways. Such enhancements by water networks have been experimentally observed.[2−4] It has thus been hypothesized that "water may be a particularly strong tunneling mediator when it occupies a sterically constrained space between redox cofactors with strong organizing forces that favor constructively interfering coupling pathways."[1]

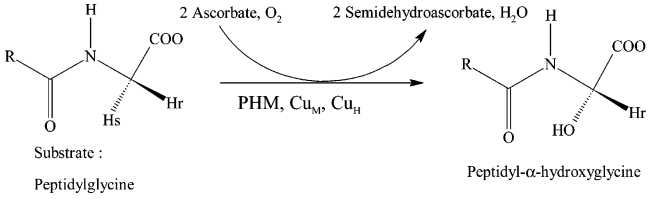
Following the previous theoretical investigations on cytochrome b_5[1] or cytochrome c_2,[5] and the very recent approaches dedicated to azurin systems,[6,7] we investigate in the present work the relevancy of the above hypothesis on the experimentally well-documented peptidylglycine α-hydroxylating monooxy-

‡ Universitat Jaume I.
# Université Pierre et Marie Curie.

(1) Lin, J.; Balabin, I. A.; Beratan, D. N. *Science* **2005**, *310*, 1311.
(2) Tezcan, F. A.; Crane, B. R.; Winkler, J. R.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5002.
(3) van Amsterdam, I. M. C.; Ubbink, M.; Einsle, O.; Messerschmidt, A.; Merli, A.; Cavazzini, D.; Rossi, G. L.; Canters, G. W. *Nat. Struct. Biol.* **2002**, *9*, 48.
(4) Francisco, W. A.; Wille, G.; Smith, A. J.; Merkler, D. J.; Klinman, J. P. *J. Am. Chem. Soc.* **2004**, *126*, 13168.
(5) Miyashita, O.; Okamura, M. Y.; Onuchic, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3558.
(6) Migliore, A.; Corni, S.; Di Felice, R.; Molinari, E. J. *Phys. Chem. B* **2007**, *111*, 3774.
(7) Bizzari, A. R.; Brunori, E.; Bonanni, B.; Cannistraro, S. *J. Mol. Recognit.* **2007**, *20*, 122.

10.1021/ja070329l CCC: $37.00

Scheme 1. The Reaction Catalyzed by PHM



genase (PHM) enzyme (enzyme identification: EC 1.14.17.3) and on its mutants.

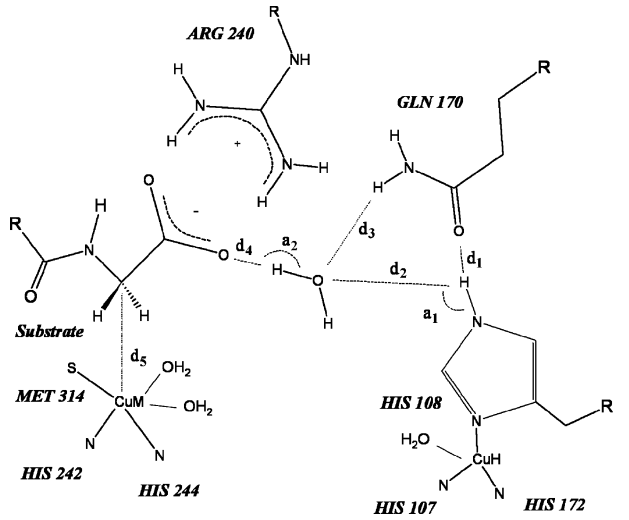
I.A. A Long Distance Electron Transfer? PHM catalyzes the stereospecific hydroxylation of the glycine α-carbon of all peptidylglycine substrates (Scheme 1).[8] Indeed this reaction is the first step toward amidation of endocrinal peptide precursors which is carried out by the bifunctional peptidylglycine-α-amidating monooxygenase (PAM) enzyme.[8,9] This amidation is the ultimate step in the biosynthesis of a majority of peptide hormones and neuropeptides which are essential for subsequent biological activity.[8,10,11] This copper- and ascorbate-dependent monooxygenase plays a major role in the activation of dioxygen and has been the subject of numerous experimental studies devoted to the understanding of its catalytic activity and, in particular, on the nature of the interaction of dioxygen with the metallic center.[4,12−21] In this regard, biomimetic systems have been developed and shown to behave as efficient modeling tools. Such an approach offers a powerful alternative to the difficulties encountered by experimental studies performed on native systems.[22−28]

Despite the intense efforts focused on the elucidation of the PHM activity, several aspects of the catalyzed peptide amidation mechanism remain unclear. In particular, the reduction of molecular oxygen into the hydroxylated product requires the electron transfer from a copper site acting as electron reservoir, namely Cu_H, to that binding O_2 and the substrate, namely Cu_M.[29] Examples of coupled binuclear copper active sites are abundant: let us mention those encountered in tyrosinase or catechol oxidase. In those cases, owing to their spatial neighboring, each of the two copper cations simply give one electron to reduce dioxygen.[30,31] By contrast, in all forms of PHM, the two metal cations are believed too far from one another to allow a direct electronic transfer,[17,32] as neither electron paramagnetic resonance (EPR) nor extended X-ray absorption fine structure (EXAFS) have detected any coupling, undoubtedly because of the solvent-filled cleft in-between.[9,15,33,34] This particularity of PHM has been confirmed by various X-ray structures showing a copper−copper distance of about 11 Å. In this regard, the long distance between the two copper cations, which is not sensitive to the binding of the substrate,[17,32] has suggested alternative mechanisms to explain the required electron transfer: (a) the use of a superoxide ion,[16] (b) the involvement of an intimate network located in-between the two active sites,[32] and (c) the participation of the substrate itself.[4,18,19,32] The superoxide mechanism was discarded by Klinman and coworkers on the basis of the relationship between O_2 consumption and substrate hydroxylation in dopamine β-monooxygenase (DβM), (enzyme identification: EC 1.14.17.1) an homologous protein believed to act like PHM.[4,35,36] The second mechanism was proposed on the basis of crystallographic data and involves an electron transfer through His108, Gln170, a water molecule, and the carboxylate group of the substrate.[8] Site-directed mutagenesis and intrinsic tryptophan fluorescence have been used to explore the role of several amino acids, especially Gln170, on active PHM, and to examine the reliability of this mechanism.[18] A note of caution has to be introduced at this point as PHM seems to be very sensitive to pH[37] and not all experiments have been conducted in the same conditions (crystallographic studies of PHM were carried out at pH 5.5, while other techniques such as EXAFS or carbon monoxide binding studies were performed at pH 7.5). The results by Bell et al. suggest that the enzyme is still active after mutating Gln170 by other residues such as Ala or Asn, but, it is inhibited when Tyr79 is replaced by Trp, although it is not involved in the proposed electron pathway.[18] The authors have suggested a substrate-mediated mechanism, bridging directly the two copper centers. Finally, more recent kinetic studies have investigated how the peptide backbone of the substrate and several water molecules between Cu_H and Cu_M could provide a pathway for the interdomain electron transfer.[4] Many studies have shown that water-mediated hydrogen bonds as well as bonds in donor−bridge−acceptor complexes are efficient ways of coupling for electron transfers, which could also take place in PHM.[38]

The above-mentioned coordination of the two metal cations appears to be a robust feature in PHM enzymes. Cu_H is coordinated by the Nδ atoms of His107, His108, and His172, while the tetrahedral Cu_M(I) cation is coordinated by the Nϵ atoms of His242 and His244, the sulfur atom of Met314, and a

(8) Prigge, S. T.; Mains, R. E.; Eipper, B. A.; Amzel, L. M. *Cell. Mol. Life Sci*. **2000**, *57*, 1236.
(9) Kulathila, R.; Merkler, K. A.; Merkler, D. J. *Nat. Prod. Rep*. **1999**, *16*, 145.
(10) Stewart, L. C.; Klinman, J. P. *Annu. Rev. Biochem*. **1988**, *57*, 551.
(11) Eipper, B. A.; Stoffers, D. A.; Mains, R. E. *Annu. Rev. Neurosci*. **1992**, *15*, 57.
(12) McGuirl, M. A.; Dooley, D. M. *Curr. Opin. Chem. Biol*. **1999**, *3*, 138.
(13) Blain, I.; Slama, P.; Giorgi, M.; Tron, T.; Reglier, M. *J. Biotechnol*. **2002**, *90*, 95.
(14) Garcia-Borron, J. C.; Solano, F. *Pigm. Cell Res*. **2002**, *15*, 162.
(15) Eipper, B. A.; Quon, A. S. W.; Mains, R. E.; Boswell, J. S.; Blackburn, N. J. *Biochemistry* **1995**, *34*, 2857.
(16) Jaron, S.; Blackburn, N. J. *Biochemistry* **1999**, *38*, 15086.
(17) Prigge, S. T.; Kolhekar, A. S.; Eipper, B. A.; Mains, R. E.; Amzel, L. M. *Science* **1997**, *278*, 1300.
(18) Bell, J.; Meskini, R. E.; D'Amato, D.; Mains, R. E.; Eipper, B. A. *Biochemistry* **2003**, *42*, 7133.
(19) Owen, T. C.; Merkler, D. J. *J. Med. Hypotheses* **2004**, *62*, 392.
(20) Prigge, S. T.; Eipper, B. A.; Mains, R. E.; Amzel, L. M. *Science* **2004**, *304*, 864.
(21) Cramer, C. J.; Tolman, W. B. *Acc. Chem. Res.* **2007**, *40*, 601−608.
(22) Gherman, B. F.; Heppner, D. E.; Tolman, W. B.; Cramer, C. J. *J. Biol. Inorg. Chem*. **2006**, *11*, 197.
(23) Lewis, E. A.; Tolman, W. B. *Chem. Rev*. **2004**, *104*, 1047.
(24) Schindler, S. *Eur. J. Inorg. Chem*. **2000**, *11*, 2311.
(25) Karlin, K. D.; Fox, S.; Nanthakumar, A.; Muthy, N. N.; Wei, N.; Obias, H. V.; Martens, C. F. *Pure & Appl. Chem.* **1995**, *67*, 289.
(26) Johansson, A. J.; Blomberg, M. R. A.; Siegbahn, P. E. R. *Inorg. Chem.* **2006**, *45*, 1491.
(27) de la Lande, A.; Gérard, H.; Moliner, V.; Izzet, G.; Reinaud, O.; Parisel, O. *J. Biol. Inorg. Chem*. **2006**, *11*, 593.
(28) de la Lande, A.; Moliner, V.; Parisel, O. *J. Chem. Phys.* **2007**, *126*, 035102.
(29) Klinman, J. P. *Chem. Rev*. **1996**, *96*, 2451 and references therein.
(30) Solomon, E. I.; Chen, P.; Metz, M.; Lee, S.-K.; Palmer, A. E. *Angew. Chem. Int. Ed*. **2001**, *40*, 4570.
(31) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem. Rev*. **1996**, *96*, 2563.
(32) Prigge, S. T.; Kolhekar, A. S.; Eipper, B. A.; Mains, R. E.; Amzel, L. M. *Nat. Struct. Biol*. **1999**, *6*, 976.
(33) Boswell, J. S.; Reedy, B. J.; Kulathila. A.; Merkler, D.; Blackburn, N. J. *Biochemistry* **1996**, *35*, 12241.
(34) Blackburn, N. J.; Rhames, F. C.; Ralle, M.; Jaron, S. *J. Biol. Inorg. Chem*. **2000**, *5*, 341.
(35) Klinman, J. P. *J. Biol. Chem*. **2006**, *281*, 3013.
(36) Evans, J. P.; Ahn, K.; Klinman, J. P. *J. Biol. Chem*. **2003**, *278*, 49691.
(37) Bauman, A. T.; Jaron, S.; Yukl, E. T.; Burchfiel, J. R.; Blackburn, N. J. *Biochemistry*, **2006**, *45*, 11140.
(38) Gray, H. B.; Winkler, J. R. *Proc. Nat. Acad. Sci. U.S.A.* **2005**, *102*, 3534.
(39) Evans, J. P.; Blackburn, N. J.; Klinman, J. P. *Biochemistry* **2006**, *45*, 15419.

Scheme 2. The Active Site of PHM (PHMcc) in Its Oxidized Form with Its Substrate[a]



[a] As deduced from X-ray crystallographic data of the oxidized form with substrate of PHMcc.[17,39] Selected distances and angles have been defined.

fourth ligand that can be a solvent molecule (Scheme 2).[20,32] From the analysis of Scheme 2, it seems that the electron-transfer channel from Cu_H to Cu_M could be established through a single water molecule, His108, and the carboxylate group of the peptide substrate. Nevertheless this possibility deserves farther attention as different techniques (mutagenesis studies, kinetic isotope effect measurements, X-ray crystallographic studies) have not rendered a unique answer to explain such a long-distance electron transfer.

I.B. The Theory for Electronic Transfers. From a theoretical point of view, the development of formalisms able to describe electron-transfer reactions began in the late 1950s. These approaches have provided a detailed description of electron-transfer reactions by means of semiclassical expressions relying on a small number of parameters.[40] The most accepted model, which relies on the Franck−Condon principle, is given in eq 1,[41] in which k_{ET} is the first-order rate constant for the electron transfer from a donor (D) to an acceptor (A), both held at given distance and orientation. This rate constant is a function of the temperature (T), the reaction driving force ($-\Delta G°$), the nuclear reorganization energy (λ), and the electronic coupling matrix element (H_{AB}) which reflects the strength of the interaction between the reactants and the products considered at the nuclear configuration of the transition state. The rate of nonadiabatic electron transfer between a donor and acceptor held at fixed distance is then:

$$k_{ET} = \sqrt{\frac{4\pi^3}{h^2 \lambda k_B T}} H_{AB}^2 \exp\left(-\frac{(\Delta G^0 + \lambda)^2}{4\lambda k_B T}\right) \qquad (1)$$

Equation 1 allows estimating the rate of electron-transfer reactions through a heterogeneous array of bounded and nonbounded contacts in polypeptide structures of proteins.[42,43]

If the electron transfer is the rate-limiting step in PHM, a reasonable assumption in view of the recent literature,[35,39,44] the ratio defined in eq 2, where $V_{max(mut)}$ and $V_{max(wt)}$ are the maximum rates of the mutant and wild-type enzymes, respectively, should be comparable with the square of the electronic coupling ratios, namely $(H_{DA})_i$:

$$\frac{V_{max(mut)}}{V_{max(wt)}} \approx \frac{(H_{DA})_{mut}^2}{(H_{DA})_{wt}^2} \qquad (2)$$

To establish eq 2 it is assumed that the $\Delta G°$ and λ involved in eq 1 remain unchanged between wild-type PHM and its mutants.

I.C. The Beratan Model. The pathway model of Beratan et al. can be used to evaluate H_{DA}.[45] Briefly, we recall that this model allows computing H_{DA} between an acceptor and a donor provided a defined electronic transfer pathway is characterized. Such a path is decomposed into subunits, depending on the nature of the bonds involved: covalent bonds, hydrogen bonds, or through-space interactions. As explained in eqs 3 to 6, a coupling decay is associated to each kind of bonds (ϵ_c, ϵ_h, and ϵ_s, for covalent, hydrogen bond, and space jump, respectively) of length R, and the total coupling is then obtained by multiplying the components relevant to each pathway:[46]

$$H_{DA} = A. \prod_{i=1}^{N_c} (\epsilon_c)_i \prod_{j=1}^{N_h} (\epsilon_h)_j \prod_{k=1}^{N_s} (\epsilon_s)_k \qquad (3)$$

where A is a prefactor.

$$\epsilon_c = 0.6 \qquad (4)$$

for each of the N_c covalent bonds,

$$\epsilon_h = \epsilon_c^{\,2} \exp[-1.7(R - 2.8)] \qquad (5)$$

for each of the N_h hydrogen bonds, and

$$\epsilon_s = \epsilon_c \exp[-1.7(R - 1.4)] \qquad (6)$$

for each of the N_s through-space jumps.

In practice, when applied to molecular dynamics (MD) simulations, a conformational averaged $\langle H_{DA}\rangle_i$ can be calculated from the probability P_i of observing a bridge through i hydrogen bonds.[47−49] The global electronic coupling $\langle H_{DA}\rangle_g$ is finally obtained as a sum over all the possible components if more than one configuration are observed:

$$\langle H_{DA}\rangle_g = \sum_i P_i \langle H_{DA}\rangle_i \qquad (7)$$

It should be noted that rate formulation in eq 7 assumes that fluctuations are rapid on the time scale of the electron transfer. This approach has shown its relevancy in a number of cases

(40) Marcus, R. A. *Adv. Chem. Phys.* **1999**, *106*, 1.
(41) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.
(42) Migliore, A.; Corni, S.; Felice, R.; Di Molinari, E. *J. Chem. Phys.* **2006**, *124*, 064501.
(43) Miller, N. E.; Wander, M. C.; Cave, R. J. *J. Chem. Phys. A* **1999**, *103*, 1084.
(44) Francisco, W. A.; Merkler, D. J.; Blackburn, N. J.; Klinman, J. P. *Biochemistry* **1998**, *37*, 8244.
(45) (a) Beratan, D. N.; Betts, J. N.; Onuchic, J. N. *Science* **1991**, *252*, 1285. (b) Betts, J. N.; Beratan, D. N.; Onuchic, J. N. *J. Am. Chem. Soc.* **1992**, *114*, 4043. (c) Curry, W. B.; Grabe, M. D.; Kurnikov, I. V.; Skourtis, S. S.; Beratan, D. N.; Regan, J. J.; Aquino, A. J. A.; Beroza, P.; Onuchic, J. N. *J. Bioenerg. Biomembr.* **1995**, *27*, 285.
(46) Tan, M-L.; Balabin, I.; Onuchic, J. N. *Biophys. J.* **2004**, *86*, 1813.
(47) Troisi, A.; Orlandi, G. *J. Chem. Phys. B* **2004**, *106*, 2093.
(48) Kurnikov, I. V.; Zusman, L. D.; Kurnikova, M. D.; Farid, R. S.; Beratan, D. N. *J. Am. Chem. Soc.* **1997**, *119*, 5690.
(49) Daizadeh, I.; Medvedev, E. S.; Struchebrukhov, A. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3703.

close to those investigated here.[1,5,50−52] Our objective is not to determine the electronic couplings accurately, but to compare the effects of various mutations on the PHM activity and to interpret them in terms of chemical perturbations of the electronic channel. This approach is justified by the available results which have shown the reliability of the pathway model to account for the structured water regime.[1] However, it should be kept in mind that the model used here does not explicitly consider multiple pathway quantum interferences. In this paper we thus report MD simulations of the PHM-substrate complex that will allow studying the fluctuations occurring at the active site of the enzyme. The possible existence of a chain of hydrogen bond interactions established between the two copper cations will be used to quantify the electron flow. The results, together with those obtained for different mutants, will be compared to experimental data and will allow predicting the most plausible mechanism for this puzzling electron-transfer step.

II. Computational Details

All calculations have been performed with the DYNAMO library program[53] using the OPLS-AA force field.[54] A parabolic potential with a force constant of 5000.00 kJ mol^{-1} Å^{-2} was used to restrain copper-ligand distances, allowing the angles and dihedral angles to relax. The charges on copper were settled at 0.3 according to previous density functional theory (DFT) calculations.[55] The resulting charge transfer (0.7 au) was distributed on the ligand atoms proportionally to their initial charge in the OPLS force field. This procedure has the advantage of not highly perturbing the force field parametrization. The van der Waals parameters for copper ($\epsilon = 0.05$ kJ/mol, $\sigma = 2.13$ Å) were taken from ref 56.

The initial coordinates of reduced PHM were obtained from the X-ray crystal structure of oxygenated PHM resolved at 1.85 Å (1SDW entry of Brookhaven Protein Data Bank).[20] The O_2 molecule coordinated at the Cu_M site in the crystal structure was simply removed. For ease of computation, the substrate used for crystallization, *N*-acetyl-diiodotyrosyl-D-threonine, was replaced by a *N*-acetyl-Phe-Gly peptide, a common PHM substrate. The hydrogen atoms were added by assigning the protonation states of all ionisable groups at pH 7 using the pK_a values of aminoacids recalculated using the "cluster method"[57] implemented by Field and co-workers[58] and according to which each titratable residue in the protein is perturbed by the electrostatic effect of the protein environment. The position of the hydrogen atoms were optimized afterward. Finally the protein was solvated in a cubic water box having a side-length of 79.5 Å. The final system thus involves about 50000 atoms.

To have the dynamic simulations more tractable, a part of the system was frozen: it includes all atoms beyond a 25 Å radius sphere centered on the crystal water molecule located in the center of the active site (ca. 43000 atoms, most of them relevant to the solvent). We have complemented this study by a fully relaxed dynamics, where no atoms were frozen during the simulation to ensure that no biases were introduced when using this model. The comparison of the trajectories obtained by means of both models renders similar results thus justifying the use of the less central processing unit (CPU) time-consuming approach in which some of the atoms are frozen.

To prepare the initial mutated enzyme structures, the mutations were operated with MOLDEN[59] from the X-ray structure of the native protein. The same protocol as that used for the wild-type enzyme was then applied.

MD simulations of 3.0 ns were carried out for the wild-type system, discarding the first 0.5 ns of the trajectories (relaxation period) to obtain time-averaged values of $<H_{DA}>_g$. The computations of electronic couplings within the pathway model framework were performed by geometrical analysis of the resulting trajectories. For mutated enzymes, MD simulations were run during 2.0 ns. Such long simulations ensure the convergence of the electronic coupling constant with respect to thermal fluctuations of the protein.[51] Time-dependent evolution of key distances (Å) for wild and mutants of PHM, as well as the electronic coupling for wild type and mutants of PHM can be found in the Supporting Information showing the robustness of the averaged results (Figures S1 and S2).

III. Results and Discussion

As explained previously, MD simulations have been performed for the wild-type PHM and for five different mutants, all of them complexed with *N*-acetyl-Phe-Gly peptide as substrate. Mutations have been carried out to investigate the role of Gln170 and Tyr79, which will be compared with available experimental data. Furthermore, from the averaged structures obtained from the dynamics, key interatomic distances have been analyzed and the relative activity of the different mutants has been evaluated by means of the previously detailed pathway model.[45]

III.A. Wild-Type PHM.

III.A.1. Structural Aspects. Figure 1 shows the time evolution along the MD simulation of some selected distances and angles of the active site (Scheme 2). The reported plots demonstrate the possible presence of a network of hydrogen bond interactions that could control the electron flow between the two copper centers. As observed, the interaction between Gln170 and His108 (measured by the distance d_1), which were at a typical hydrogen-bond length in the initial structure, is rapidly weakened after 0.6 ns and a new interaction with a water molecule is established (d_2), concertedly. Another important change concerns the interaction between this water molecule and Gln170 (d_3): while the oxygen atom of the solvent molecule and the N atom of Gln were far away (ca. 5 Å) in the starting structure derived from X-ray data, a hydrogen bond is established between both species after 0.4 ns of MD. Furthermore, this water molecule strongly interacts with the carboxylate group of the substrate (d_4), which consequently results in constraining it in the cavity and supports its dramatic role in an electron flow path. The time evolution of the two angles a_1 and a_2 is also consistent with the formation of the two hydrogen bonds mentioned above: although more dramatically eye-catching in the a_2 case, the converged values of the angles are in better accordance, for both cases, with the expected values for standard N−H−O or O−H−O hydrogen bonds than in the starting geometry. The distance between Cu_M and the aliphatic hydrogen atom of the substrate (d_5) shortens slightly from 4.5 to 3.8 Å

(50) Miller, N. E.; Wander, M. C.; Cave, R. J. *J. Phys. Chem. A* **1999**, *103*, 1084.
(51) Skourtis, S. S.; Balabin, I. A.; Kawatsu, T.; Beratan, D. N. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3552.
(52) Prytkova, T. R.; Beratan, D. N.; Skourtis, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 802.
(53) (a) Field, M. J. *A Practical Introduction to the Simulation of Molecular Systems*; Cambridge University Press: Cambridge, U.K., 1999. (b) Field, M. J.; Albe, M.; Bret, C.; Proust-de Martin, F.; Thomas, A. *J. Comput. Chem.* **2000**, *21*, 1088.
(54) Jorgensen, W. L.; Maxwell, D. S.; Tirado-Rives, J. *J. Am. Chem. Soc.* **1996**, *118*, 11225.
(55) Comba, P.; Remenyi, R. *J. Comput. Chem.* **2002**, *23*, 697.
(56) Buning, C.; Comba, P. *Eur. J. Inorg. Chem.* **2000**, 1267.
(57) Antosiewicz, J.; McCammon, J. A.; Gilson, M. K. *J. Mol. Biol.* **1994**, *238*, 415.
(58) Field, M. J.; Amara, P.; David, L.; Rinaldo, D., personal communication.
(59) Schaftenaar, G.; Noordik, J. H. *J. Comput.-Aided Mol. Design* **2000**, *14*, 123.

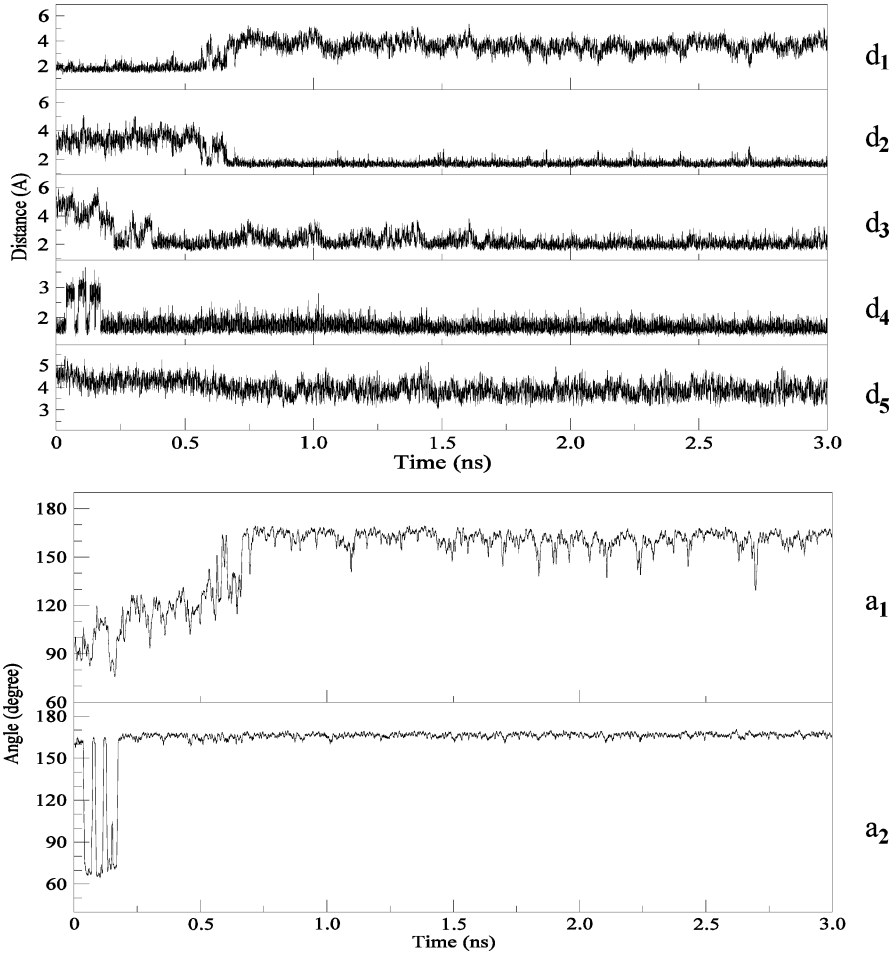


Figure 1. Time-dependent evolution of key distances (Å) and angles (deg) depicted in Scheme 2 for wild PHM.

after the first 1 ns of the simulation, remaining then closer to the copper cation during the rest of the MD simulation. The final PHM−substrate complex obtained after a 3.0 ns MD simulation is shown in Figure 2a. From the analysis of this structure, together with the results presented in Figure 1, it is possible to predict that an electron pathway can be drawn between the two copper cations through His108, a water molecule, and the substrate (Scheme 2). A similar kind of pathway has been proposed on the basis of frozen protein crystals soaked with a slow substrate and ascorbate in the presence of dioxygen.[20] The role of other important residues such as Gln170 and Arg240 in fixing the positions of the different moieties of the active site in a proper orientation to facilitate the electron transfer is especially important in the case of the mobile water molecule.

III.A.2. Kinetic Aspects. To quantify the rate constant for a possible electron transfer through the pathway described above, the global electronic coupling $\langle H_{DA}\rangle_g$ (eqs 3 and 7) has been computed using the semiclassical model of Beratan for two different structures: the X-ray structure and the one obtained as an average over the last 2.5 ns of the MD simulations. The ratio between the two obtained values amounts to 44 in favor of the latter. It thus appears that the structure of the protein obtained after the MD simulation is better prepared for the electron flow through the different hydrogen and covalent bonds occurring in the donor−bridge−acceptor complex than the original X-ray structure. Furthermore, the analysis of other PHM X-ray crystallographic structures[8,17,20,60] reveals the presence of a robust hydrogen bond between His108 and Gln170 (d_1 in Scheme 2) which is competing with an interaction with the water molecule. These results justify the need of running proper MD simulations to obtain a realistic picture of the protein, the flexible character of which can be hindered if only crystallographic data are analyzed.

To support the hypothesis of an electron-transfer pathway the evaluation of $\langle H_{DA}\rangle_g$ for different mutants can be done. Furthermore, a comparison of theoretically predicted relative electron-transfer rate constants with experimentally measured ones can be carried out, and the role of the different residues at the active site of the enzyme can be analyzed. Thus, accepting this hypothesis of an electron path, deviations of d_1, d_2, and d_3 from the optimized values converged after MD simulations should reduce the enzyme activity as will be illustrated below.

III.B. Mutants. To confirm the importance of the coupling through covalent and hydrogen bonds in the long-range electron transfer, several mutants have been investigated. The corresponding results have then been compared to those related to the native PHM. In particular, Gln170 was mutated to Asn and Glu, structurally related aminoacids, to Leu, a hydrophobic residue, or to Ala, a much smaller nonpolar amino acid. Tyr79, which has been postulated to assist in the channeling of superoxide formed at Cu_A toward Cu_M,[16] was mutated to Trp

(60) Siebert, X.; Eipper, B. A.; Mains, R. E.; Prigge, S. T.; Blackburn, N. J.; Amzel, L. M. *Biophys. J.* **2005**, *89*, 3312.

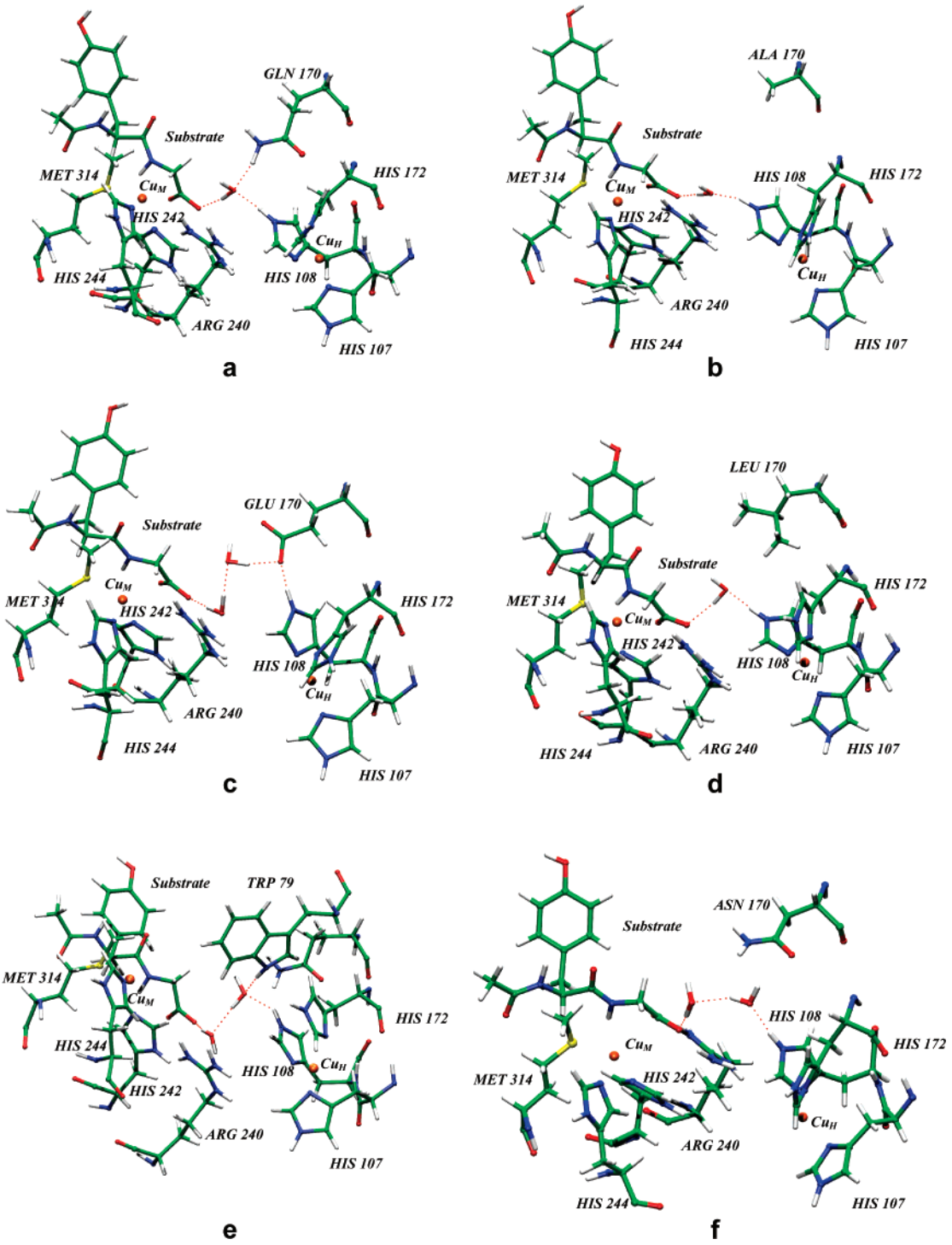


Figure 2. Detailed structure of the active sites in their optimal orientation for the long-range electron-transfer obtained from the MD simulation performed on (a) wild-type PHM, (b) Gln170Ala, (c) Gln170Glu, (d) Gln170Leu, (e) Tyr79Trp, and (f) Gln170Asn.

to evaluate the effect of having a different, larger, and hydrophobic residue with no hydroxyl group.

To compute $\langle H_{DA} \rangle_i$, the existence of the channel between the carboxylate termination of the substrate and His108 was checked at every step of the MD simulation and H_{DA} evaluated when relevant, namely, if the distance between the appropriate entities was found about a standard value corresponding to the kind of bond under analysis. It must be pointed out that only the channel between the carboxylate group of the substrate and His108 was examined as its contribution has to be much larger than that induced by space jumps. The relative enzyme activity, together with the conformational averaged electron coupling, $\langle H_{DA} \rangle_i$, and the global electronic coupling, $\langle H_{DA} \rangle_g$, computed according to eq 7, is given in Table 1. Time-dependent evolution of key distances (Å) and angles (deg) depicted in Figure 2 for mutants of PHM is reported in the Supporting Information.

The results for the different mutations are as follows. The **Gln170Ala** mutation implies the replacement of a residue which constrains the bridging water molecule in a proper orientation, by a smaller residue. As observed in Figure 2b, the smaller size

Table 1. The PHM Activity of Various Mutants as a Function of the Number of Hydrogen Bonds Established in the Bridge, *i*, the Probability of Hydrogen Bond Formation Measured as a Frequency during the Simulation, P_i, and the Electronic Couplings $\langle H_{DA}\rangle_i$ and $\langle H_{DA}\rangle_g$

						$(V_{max(mut)}/V_{max(wt)})_{exp}$	
PHM sample	*i*	P_i	$1/A\langle H_{DA}\rangle_i$	$1/A\langle H_{DA}\rangle_g$	$\langle H_{DA}\rangle_g^2/\langle H_{DA}\rangle_{wt}^2$	A[b]	B[c]
wild type	2	0.94	1.64E-02	1.54E-02	1.00	1	1
Gln170Asn	3	0.163	6.75E-03	1.10E-03	0.01	0.85	
Gln170Ala	2	0.83	1.57E-02	1.30E-02	0.71	1.26	1.16/0.8
Gln170Glu	4	0.72	3.50E-03	2.52E-03	0.03	0.07	
Gln170Leu	2/3	0.13/0.02	1.35E-02/6.62E-03	1.89E-03	0.01	0.05	
Tyr79Trp	2/3	0.04/0.37	1.16E-02/6.89E-03	3.01E-03	0.04	0.02	0.007/0.005

[a] The experimental values of the maximum rates come from ref 18. PHMcc refers to the so-called PHM catalytic core (residues 42 to 356 only). [b] Experimental values obtained by means of transiently expressed PHMcc proteins at pH = 7 (see ref 18 for details). [c] Experimental values obtained by means of purified PHMcc proteins at two different pH values: 7.0 and 5.5 (see ref 18 for details).

of Ala favors the compression of the active site, thus slightly shortening, from 4.51 to 4.42 Å, the distance between the carboxylate oxygen atom of the substrate and the nitrogen atom of His108. Subsequently, the water molecule is still fixed between these atoms. The probability of finding this structure is close to that estimated for the wild type (0.83, Table 1), which explains the experimentally measured high efficiency of this mutated enzyme. This computation thus highlights the contribution of Gln170 in maintaining the water molecule as its replacements by Ala170 induce a small loss of activity.

When Gln170 is replaced by Glu to get the **Gln170Glu** mutant, the effect of the negatively charged carboxylate group of the new amino acid provokes a disruption in the active site structure. Now the electron-transfer channel is made by two water molecules (Figure 2c), with a significant probability (0.72) of finding this structure during the MD simulation. However, the electron transfer through more than one water molecule appears to be much slower, as observed experimentally and predicted with our model (Table 1).

The exchange of Gln170 by a hydrophobic residue, which corresponds to the **Gln170Leu** mutation, slightly opens the cavity. This, together with the fact that no hydrogen bond can be established between the residue and the bridging water molecule, makes that, although a possible electron channel is observed (Figure 2d), the probability of finding this structure is very low (0.13). The result is that the predicted efficiency of the mutant is dramatically reduced, as observed experimentally (Table 1). Another kind of bridge, which involves two water molecules, has been observed during the MD simulations, but this structure presents an even lower probability (0.02). Consequently, although the global electronic coupling will be the sum of two terms for this mutant, their low probabilities, together with the fact that the efficiency of long bridge must be very low, explains the behavior of this mutant.

A similar situation is observed in the **Tyr79Trp** mutant where, again, two kinds of bridge through one or two water molecules are observed during the MD simulation (Figure 2e). Still, these structures present a low probability: 0.04 and 0.37, respectively. The remaining activity of this sample can be explained by the effect of the Trp residue: the electron acceptor group of the ring, the polar −NH group, interacts with the water molecule and helps in retaining, at some extent, a favorable orientation.

Finally, the **Gln170Asn** mutant appears as a singular case. By comparison with the wild-type enzyme, Asn presents the same functional group as Gln but a shorter carbon chain (one carbon atom less). The consequence is that the new residue is located far from the substrate, the water molecule, and His108. The hydrogen-bond interaction with the water molecule is still possible, albeit pushing it away from the initial bridge. A hole is created, which can be occupied by a second water molecule. Nevertheless, this situation, depicted in Figure 2f, does not appear with high frequency during the MD simulation (P_i = 0.163). The resulting predicted efficiency of the mutant is consequently very low, in contrast to the results reported by Bell et al.[18] A note of caution has to be introduced at this point. First, the activity of both, enzyme and mutants, are dramatically reduced at pH = 7.0 by comparison to the values obtained at pH 5.5. Furthermore, the experimental errors (standard deviations) reported by the authors are quite large (more than 50%). Finally, dramatically different relative V_{max} values are obtained for the **Gln170Ala** or **Tyr79Trp** mutants, depending on the experimental approach and pH considered (see Table 1 and reference 11). These considerations could explain, at least partly, the discrepancy between our theoretical prediction and the experimental results for **Gln170Ala** and **Tyr79Trp**, suggesting that the data to be compared with our results are those obtained at pH 5.5.

IV. Conclusions

In this paper we have reported long MD simulations for the wild-type PHM and five different mutants, all of them complexed with a Phe-Gly peptide as a substrate. The possible electron-transfer pathway established between the long-distance two copper I cations of the enzyme active site has been analyzed. Its efficiency has been estimated for each case by means of the semiclassical approach of Beratan and compared with experimental data.

The results show that an electron flow can take place through a channel of covalent and hydrogen bonds established between the two copper cations. The carboxylate group of the substrate, a water molecule, and His108 are expected to be the bricks of this bridge. Our simulations reveal that MD are required to obtain a reliable configuration of these species that optimize the electron channel: conclusions derived from crystallographic structures only could hide important information. Furthermore, other residues such as Gln170 and Arg240 have been shown to play a key role by fixing the flexible water molecule in a proper orientation. This key feature confirms the previous hypothesis by Lin, Balabin, and Beratan that enzymes could organize this kind of water-mediated electron transfer.[1]

From averaged structures obtained during the dynamics performed on the different mutants it has been evidenced that the enzyme efficiency is related to the establishment of the

above-mentioned network of hydrogen and covalent bonds. This theoretical prediction has been quantitatively estimated from the number of hydrogen bonds, their probability of formation, and the electronic coupling. The results show a very good agreement with experimental measurements, thus confirming the reliability of the calculations. In particular, we observe a good agreement between the experimentally determined relative maximum rate of the mutants and the predicted square of the electronic couplings, with the exception of Gln170Asn. Overall, our results demonstrate the possibility of an electron-transfer pathway through a network of hydrogen and covalent bonds, as well as the role of the residues at the active site. They also provide another support to the hypothesis that the long-range electron transfer might be the rate-limiting step in PHM-catalyzed reactions. The pathway gained from the PHM study could be extended to DβM, giving for example residue Glu268 the same role as Gln170 in PHM.[8,61]

Acknowledgment. We thank the DGI for Projects DGI CTQ2006-15447-C02-01/BQU, UJI-BANCAIXA foundation for Project P1·1B2005-13 and Generalitat Valenciana for Project GV06/152. We acknowledge the Servei d'Informàtica of the Universitat Jaume I (Spain), the CCRE at Université Pierre et Marie Curie (France), the French IDRIS (Orsay, France), CINES (Montpellier, France), and CRIHAN (Saint-Etienne-du-Rouvray, France) supercomputer centers for providing us with computer capabilities.

Supporting Information Available: Time-dependent evolutions of key distances (Å) (Figure S1) and electronic couplings (Figure S2) for wild-type and mutants PHM. This material is available free of charge via the Internet at http://pubs.acs.org.

JA070329L

(61) Kamashi, T.; Kihara, N.; Shiota, Y.; Yoshisawa, K. *Inorg. Chem.* **2005**, *44*, 4226.