Theoretical study of phenol and 2-aminophenol docking at a model of the tyrosinase active site

Jean-Philip Piquemal,[a] Jacques Maddaluno,[b] Bernard Silvi[a] and Claude Giessner-Prettre[a]

[a] *Laboratoire de Chimie Théorique, Université Pierre & Marie Curie, Case Courrier 137, 4, place Jussieu, 75252, Paris cedex 05, France. E-mail: cgp@lct.jussieu.fr; Fax: +33 144 275 526; Tel: +33 144 272 504*

[b] *Laboratoire des Fonctions Azotées & Oxygénées Complexes de l'IRCOF, Université de Rouen, 76821, Mont Saint Aignan cedex, France. E-mail: jmaddalu@crihan.fr; Fax: +33 235 522 446; Tel: +33 235 522 971*

Received (in Montpellier, France) 21st October 2002, Accepted 18th February 2003
First published as an Advance Article on the web 15th May 2003

DFT calculations using the B3LYP functional are reported for a model of the (oxy)tyrosinase active site including the two copper cations, six imidazoles and dioxygen (in the oxy form), plus either phenol (taken as a model of tyrosine, the enzyme's natural substrate) or 2-aminophenol, a very efficient inhibitor. The results obtained suggest that (i) both the substrate and the inhibitor have to be deprotonated to form a stable complex with the model of the "native" form of the enzyme; (ii) only phenol binds to the oxy form; (iii) the complex formed between our oxy model and 2-aminophenate is more stable than that with phenate, suggesting a competitive inhibition mechanism between the deprotonated forms of the substrate and of the inhibitor.

Introduction

Tyrosinase is a widespread dinuclear copper enzyme present in microorganisms as well as in plants and mammals. It is able to oxidize most phenols to the corresponding catechols and, eventually, *o*-quinones.[1] Albeit it has been studied for quite a while, this enzyme and its models have recently become the subjects of a rapidly increasing number of experimental[2] and theoretical[3] investigations. Both approaches aim to understand the enzyme's mechanism of action, of which very little is known to date. Most of the information available has been obtained from structural and functional models. Among the relevant data, let us mention the results obtained by Karlin *et al.*[4] suggesting that ring oxidation takes place through an electrophilic mechanism. Simultaneously, Casella *et al.*[5] have observed that the substrate can undergo oxidation under its deprotonated (phenate) form. Recent experimental evidence obtained from tyrosinase and catechol oxidase inhibitors[2c,6] as well as model systems[2e,f] fully support this assumption.

On the other hand, several experimental studies have shown that subsituted phenols, such as 2-aminophenol[7] and 4-nitrophenol,[8] or benzene derivatives, such as benzoic and naphthoic acids,[8] can be efficient tyrosinase inhibitors. Because of the similarity between the structures of these compounds and those of phenol or tyrosine, one can reasonably suppose that the inhibition takes place through competitive docking. To test the validity of this hypothesis, we decided to undertake a series of computations on several complexes that could be formed between models of the native (deoxy) and of the oxy forms of tyrosinase, that is in the absence (or presence) of dioxygen, and phenol (or phenate). The same set of calculations was repeated replacing phenol by an inhibitor. We retained for this study one of the most efficient tyrosinase inhibitors described in the literature,[7] 2-aminophenol (2-AP), keeping in mind that it is oxidized into *o*-quinone imines upon prolonged contact.[9] Obviously, this work does not encompass all tyrosinase inhibition modes such as Schiff base formation known to occur with aromatic aldehydes.[2a] Because of the different experimental data[2c,e,f,5,6] showing that oxidation could occur on the deprotonated form of the substrate, the same series of computations was also carried out for the most stable anions of phenol and 2-aminophenol.

The determination of a similar docking for the substrate on the one hand and the inhibitor on the other, with an interaction energy in favor of the latter, would present a twofold interest, that is provide an interpretation of the inhibition phenomenon and information on the binding mode of the phenolic substrate at the enzyme active site.

Results and discussion

We have considered in our calculations an active site model including the two copper cations, each coordinated to three imidazoles $\{[Cu^{+}(ImH)_3]_2$, hereafter called TYR[6b]$\}$, as retained by Siegbahn and Wirstam,[3d] since experimental data indicate the presence of six histidines in the tyrosinase active site.[10] The complete geometry optimizations were carried out with Jaguar 4.1[11] using the B3LYP functional[12] and the LACVP** basis set. Zero-point vibrational effects (ZPVE) and the basis set superposition error (BSSE) corrections are included in the reported interaction energies.

The TYR native form model (*i.e.*, without dioxygen), our reference, corresponds to the most stable $Cu^{+}(ImH)_3$ dimer. This optimized "metastable" complex has an energy value of −1749.4145 a.u. and a Cu–Cu distance of 3.0 Å(Tables 1 and 2). Previous results have shown that such arrangements correspond to local energy minima, despite the presence of a positive charge on the two moieties of the system.[13] The origin of this local stability is not rooted in the copper–copper bond, as indicated by the electron localization function (ELF) analysis[14] of a $[Cu^{+}(ImH)_3]_2$ complex, with an "enzyme-like" Cu–Cu distance of 3.6 Å,[15] which does not exhibit any valence basin between the copper nuclei, even at very low ELF values (Fig. 1).

Table 1 Energy values and zero point vibrational energy (ZPVE) of the interacting entities considered

	E/a.u.	ZPVE/kcal·mol^{-1}
$(Cu^{+}(ImH)_3)_2$	−1749.4145	322.6
$(Cu^{+}(ImH)_3)_2 - O_2$	−1899.7363	321.4
Phenol	−307.4725	71.9
Phenate	−306.8861	63.0
2-Aminophenol	−362.8298	82.5
2-Aminophenate	−362.2474	73.3

On the other hand, a CSOV[16] (Constrained Space Orbital Variation) analysis of the interaction energy between the two $Cu^{+}(ImH)_3$ entities[17] shows that, with increasing Cu–Cu distances, the electrostatic repulsion decreases more slowly than the attractive polarization and charge transfer contributions. This difference is responsible for the existence of local minima on the overall repulsive energy surface.

The TYR model has been considered first, together with its complexes with either phenol or 2-aminophenol. The optimization leads to the dissociation of the model into its two monomers, as can be seen from Fig. 2. The Cu–Cu distance becomes larger than 9 Å, a feature hardly compatible with an enzyme active site. This result is supported by experimental data on tyrosinase functional models that do not form stable complexes with phenol.[18] A similar low binding affinity of the substrate, which has been reported for the Cu(I)–Cu(I) center of catechol oxydase,[6a] is also in agreement with our result. The calculated values of the interaction energy of the optimized $Cu^{+}(ImH)_3$ dimer is −5.3 kcal·mol^{-1} with phenol and −15.2 kcal·mol^{-1} with 2-AP. The latter value is due to the larger interaction energy of Cu^{+} with nitrogen than with oxygen,[12a] leading to a shorter distance between Cu and the inhibitor as can be seen in Fig. 2(B). For these systems we did not calculate the ZPVE and BSSE contributions to the binding energy since these quantitites would be meaningless for such "dissociated complexes".

By contrast, the optimized arrangements of the complexes formed between TYR and the deprotonated forms of the substrate (phenate) and of the inhibitor (2-AP-O^{-}) depicted in Fig. 3 appear reasonable. The Cu–Cu distances are of 3.9 and 4.3 Å (Table 2) in the TYR–phenate and TYR–2AP-O^{-} complexes, respectively. These values are within the experimental range since distances from 2.87 Å in the oxydized form of a catechol oxidase[6a] to 4.6 Å in the native form of an hemocyanin[19] have been measured for such binuclear centers. The most striking feature of these calculated structures is the location of the substrate/inhibitor between the two coppers. Such a type of structure is in agreement with NMR data obtained for tyrosinase functional models and with X-ray data concerning the complex formed between a catechol oxidase and the inhibitor phenylthiourea.[6a] In the former case the two Cu(I) environments remain equivalent upon substrate binding[5a] while in the latter the sulfur of the inhibitor phenylthiourea

Table 2 Optimized geometrical parameters.

	d(Cu–Cu)/Å	d(Cu–O)[a] /Å	d(O···HX)[b] /Å	φ(CuOOCu)[c] /°
TYR	3.00	—	—	—
TYR–O_2	3.56	—	—	135.
TYR–phenate	3.93	2.21/2.22	—	—
TYR–2-AP-O^{-}	4.61	2.28/2.28	—	—
TYR–O_2–phenol	3.87	3.60/3.86	1.70	167.
TYR–O_2–2-AP-OH	3.84	3.47/3.68	1.76	160.
TYR–O_2–phenate	3.33	3.97/4.17	–	119.
TYR–O_2–2-AP-O^{-}	4.31	3.54/5.31	1.72	163.

[a] Distances between the copper cation and the substrate/inhibitor oxygen atoms. [b] Distance between the OH/NH proton and the closest atom of dioxygen. [c] Dihedral angle measuring the "butterfly" effect.

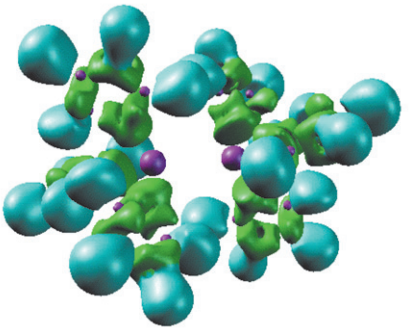

Fig. 1 ELF function localization domains ($\eta = 0.7$) for a "standard" $[Cu^{+}(ImH)_3]_2$ complex. Color code is as follows: magenta = core, green = valence disynaptic, blue = hydrogens

is coordinated to both catechol oxidase copper ions.[6a] Worthy of note is the overall difference between the imidazole arrangement in our theoretical model of these two complexes. We see from the small inlay in Fig. 3(B) that in the assembly with 2-AP-O^{-} the six ligands are perfectly staggered while this is definitely not the case for that with phenate [Fig. 3(A) inlay]. This difference can be tentatively assigned to the variation in the Cu – Cu distance between the two complexes. The larger value calculated for the complex including 2-AP-O^{-}, which can be ascribed to the steric hindrance of the amino group, prevents π-stacking between two of the imidazoles as obtained for the complex with phenate. The calculated interaction energies are one order of magnitude larger (−134.0 and −131.3 kcal·mol^{-1}, respectively) than the preceding ones (Table 3). However, these values do not include the deprotonation energies of phenol and 2-AP, which are respectively 359.1 and 355.3 kcal·mol^{-1}, as can be seen from Table 1 and in ref. 17. At this point, the overall energy slightly favors the 2-aminophenate. However, the difference between the two complexes (1.1 kcal·mol^{-1}) is small enough to be reversed upon dioxygen fixation.

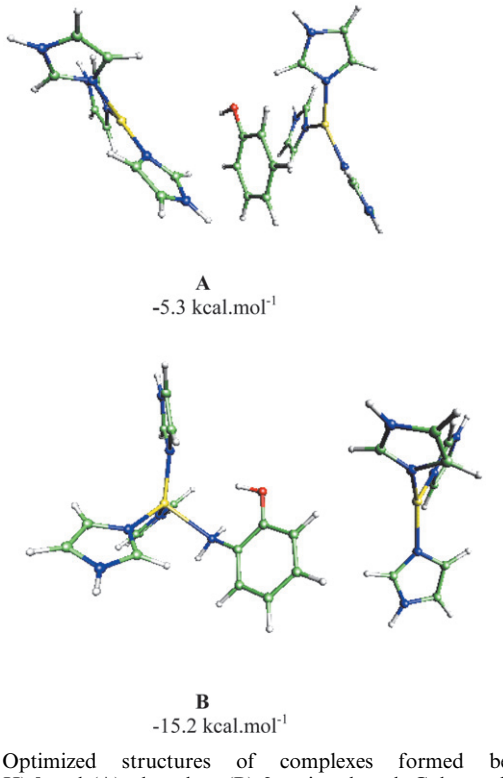

Fig. 2 Optimized structures of complexes formed between $[Cu^{+}(ImH)_3]_2$and (A) phenol or (B) 2-aminophenol. Color code is as follows: green = C, blue = N, red = O, white = H, yellow = Cu.

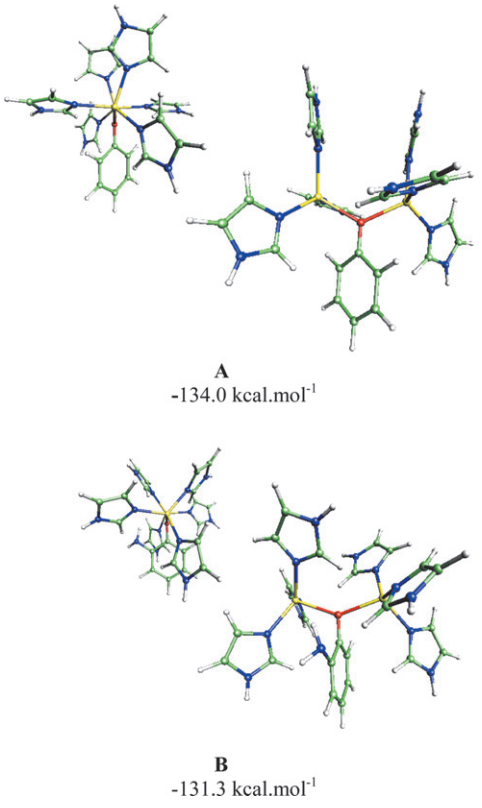

Fig. 3 Optimized structures of the complexes formed between $[Cu^{+}(ImH)_3]_2$and (A) phenate or (B) 2-aminophenate. Color code as in Fig. 2.

Let us now consider the $[(Cu^{+}(ImH)_3]_2–O_2$ system (hereafter called TYR–O_2), the model used for oxytyrosinase. We have to stress here that only the $\mu–\eta^2:\eta^2$ binding mode was considered in this study since previous results tend to show that the $(\mu–O)_2$ arrangement is not relevant to exogenous phenol oxidation by tyrosinase. The various pieces of evidences are as follows: (i) the $\mu–\eta^2:\eta^2$ binding mode is the only one observed when copper ligands are imidazoles,[20] as in the present case; (ii) to the best of our knowledge, only endogenous ligand CH bonds have been experimentally oxidized by $(\mu–O)_2$ systems;[21] (iii) previous calculations on the oxytyrosinase active site model that we are using have led to an energy difference of 23.3 kcal·mol^{-1} in favor of the $\mu–\eta^2:\eta^2$ arrangement;[3d,22] (iv) for two very similar oxytyrosinase models, one adopting the $\mu–\eta^2:\eta^2$ dioxygen binding mode and the other exhibiting an equilibrium between the μ-$\eta^2:\eta^2$ and $(\mu$-$O)_2$ geometries, it has been shown that only the former is able to hydroxylate phenates in appreciable yields.[2f]

The optimized structure in the absence of substrate/inhibitor has Cu–Cu and O–O distances that are, as noticed previously,[3d] in fine agreement with experimental data concerning oyxhemocyanin (3.56 *vs.* 3.59 Å and 1.40 *vs.* 1.41 Å , respectively). A poorer agreement is reached between the calculated and experimental φ(CuOOCu) angle values (156° *vs.* 135°), most probably due to the unsymmetrical arrangement of dioxygen between the two oxyhemocyanin copper cations. Also, the computations give, for the "remote" ligand, Cu–N distances shorter than the measured ones (2.21 and 2.24 Å *vs.* 2.40 and 2.44 Å).

The structures calculated for the complexes between TYR–O_2 and phenol as well as 2-AP are reported on Fig. 4. As above, both the substrate and the inhibitor are located between the two coppers. In the case of phenol this result is quite different from the arrangement studied by Siegbahn,[3d,e] in which the phenol is directly coordinated to one of the coppers. When starting with phenol bound to one of the two coppers the optimization yields, in most cases, to structures in which phenol oxygen is approximately equidistant from both metal cations. The Cu–Cu distances of 3.9 and 3.8 Å calculated for the optimized phenol and 2-AP complexes, respectively, are now somewhat larger than that found by Magnus *et al.*[19] for oxyhemocyanin or that calculated when phenol binds to only one copper (3.5 Å),[3d] but shorter than that measured in the complex between catechol oxydase and phenylthiourea (4.2 Å).[6a] Therefore, the distances we calculate appear reasonable and tend to show that the presence of dioxygen is a determining factor to ensure cohesion of the active site upon phenol approach. At this point it is worth mentioning that the complex with "butterfly" folding away from phenol and not toward it as on Fig. 4(A) is less stable by 4 kcal·mol^{-1}. But more important in relation with the subject of this work are the energy values reported in Table 3 and on Fig. 4, which show that 2-AP will not bind to TYR–O_2 . By contrast we calculate a binding energy larger than 5 kcal·mol^{-1} for phenol. The repulsive character of the TYR–O_2–2-AP complex is due to the large value of the sum of the zero point vibrational contribution (6.0 kcal·mol^{-1}) plus the BSSE correction (7.0 kcal·mol^{-1}). This last term would most probably be much smaller if a larger basis set were used but it would not change the qualitative relative affinities of the two substrates since the sole difference between the total energies of the two complexes plus the ZPVE contribution is in favor of phenol by 6.0 kcal·mol^{-1}. Finally, the examination of the conformations of these complexes show that the strong hydrogen bond appearing between the phenol OH proton and one oxygen of O_2 is tighter with phenol than with 2-AP (1.70 *vs.* 1.76 Å, Table 2). This difference certainly contributes to the greater stability of the phenol complex.

The optimized forms of the last systems considered, that is TYR–O_2 plus phenate or 2-AP-O^{-}, are displayed on Fig. 5. As in the two previous cases the phenates are located between the two $Cu^{+}(ImH)_3$ groups but the orientation of the 2-AP-O^{-} amino group is significantly modified with respect to that obtained for the complex between $[(Cu^{+}(ImH)_3]_2–O_2$ and 2-AP.

Table 3 Values of the different contributions to the complexation energy

Complex	E/a.u.	ΔE/ kcal·mol^{-1}	ZPVE/ kcal·mol^{-1}	ΔZPVE/ kcal·mol^{-1}	BSSE/ kcal·mol^{-1}	ΔE_{tot}/ kcal·mol^{-1}	$\delta\Delta E_{tot}$[a]/ kcal·mol^{-1}
TYR–phenol	−2056.9013	−5.3	[b]	—	—	−5.3	−9.9
TYR–2-AP-OH	−2112.2760	−15.2	[b]	[b]	[b]	−15.2	
TYR–phenate	−2056.5443	−149.0	380.2	0.7	14.3	−134.0	2.7
TYR–2-AP-O^{-}	2111.9110	−151.7	394.8	4.6	15.8	−131.3	
TYR–O_2–phenol	−2207.2274	−13.1	399.3	6.1	1.8	−5.2	11.0
TYR–O_2–2-AP-OH	2262.5825	−7.2	410.0	6.0	7.0	5.8	
TYR–O_2–phenate	−2206.8612	−147.2	395.1	10.7	13.2	−123.6	−25.0
TYR–O_2–2-AP-O^{-}	−2262.2310	−152.2	400.6	4.9	3.6	−148.6	

[a] $\delta\Delta E_{tot} = \Delta E_{tot}$(inhibitor) − ΔE_{tot}(substrate). [b] Not calculated.

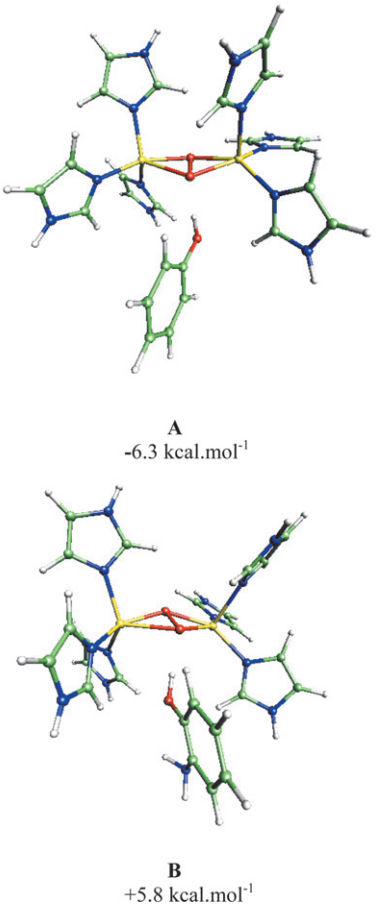

Fig. 4 Optimized structures of the complexes formed between $[Cu^{+}(ImH)_3]_2$–O_2 and (A) phenol or (B) 2-aminophenol. Color code as in Fig. 2.

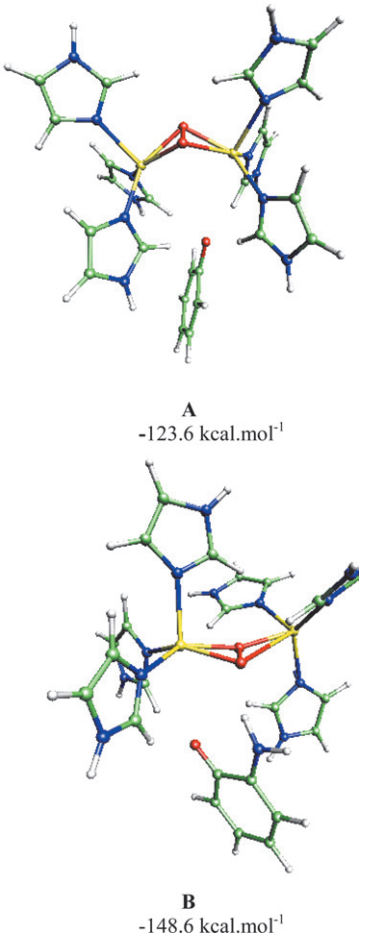

Fig. 5 Optimized structures of the complexes formed between $[Cu^{+}(ImH)_3]_2$–O_2 and (A) phenate or (B) 2-aminophenate. Color code as in Fig. 2.

This difference is due to the occurence of a hydrogen bond between dioxygen and one of the amino group protons, now the only ones available for such an interaction. The values in Table 2 show also that the binding of phenate to TYR–O_2 is associated to a particularly important butterfly effect (119°), larger than that generated by phenol (167°) or 2-AP-O^- (163°) complexation. This out-of-plane deviation is directed away from the substrate and is associated to a shorter Cu–Cu distance. On the contrary, in the case of the 2-AP-O^- complex, the butterfly bends toward the inhibitor and the Cu–Cu distance increases to 4.31 Å, probably because of the amino group steric hindrance again. The interaction energy values, reported in Table 3, show that the complex with 2-AP-O^- is 25 kcal·mol^{-1} more stable than the one with phenate. Therefore, the presence of 2-AP-O^- should inhibit the substrate binding. This energy gap is further increased when taking into account the difference between phenol and 2-AP deprotonation. Thus, a phenate anion will not be able to bind at the active site if a 2-AP-O^- anion is present. The structure obtained for the TYR–O_2–2-AP-O^- complex also indicates that the inhibiting power of a molecule will increase with its acidity and the H bonding donor capability of its anionic form.

Concluding remarks

This set of results tends to show that the inhibition due to 2-AP takes place through a competitive binding between the deprotonated (amino)phenate forms. It implies also that the oxidation of the enzyme substrate, that is tyrosine, takes place on its phenate form. This feature is supported by from recent data on a model system that has been shown to rapidly hydroxylate sodium and lithium phenates and not to react with phenol itself.[2f] Thus, the fate of the phenolic proton could have a major effect on the inhibition process. A basic residue is likely to scavenge this relatively acidic proton while phenol or 2-AP approach the copper cations. Thus, a carboxylate moiety (as found on Asp or Glu residues) in close proximity to the active site could represent an appropriate protonation site. Such a mechanism has been proposed for a plant catechol oxidase, the proton acceptor being a glutamic residue located close to the active site.[6a]

The relevance of intermediates such as those we have characterized to tyrosinase inhibition is currently being evaluated by extending this study to several inhibitors, among those described by Conrad *et al.*[8] An analysis of the system involving two Cu(II) cations, mimicking the native *met* form of tyrosinase, will also be proposed.

Acknowledgements

We thank both the Institut du Developpement et des Ressources en Informatique Scientifique (IDRIS, Orsay) and the Centre de Ressources Informatiques de Haute-Normandie (CRIHAN, St Etienne-du-Rouvray) for computer time.

References

1 (*a*) K. Lerch, *Life Chem. Rep.*, 1987, **5**, 221; (*b*) E. I. Solomon, P. Chen, M. Metz, S. -K. Lee and A. E. Palmer, *Angew. Chem., Int. Ed.*, 2001, **40**, 4570.
2 *Inter alia:* (*a*) A. Poma, G. Pacioni, S. Colafarina and M. Miranda, *FEMS Microbiol. Lett.*, 1999, **180**, 69; (*b*) L. Bubacco,

E. Vijgenboom, C. Gobin, A. W. J. W. Tepper, J. Salgado, G. W. Canters, *J. Mol. Catal. B: Enz.*, 2000, **8**, 27. (*c*) M. van Gastel, L. Bubacco, E. J. J. Groenen, E. Vijgenboom and G. W. Canters, *FEBS Lett.*, 2000, **474**, 228; (*d*) M. Jiménez, S. Chazzara, J. Escribano, J. Cabanes and F. Garcia-Carmona, *J. Agric. Food. Chem.*, 2001, **49**, 4060; (*e*) S. Itoh, H. Kumei, M. Taki, S. Nagatomo, T. Kitagawa and S. Fukuzumi, *J. Am. Chem. Soc.*, 2001, **123**, 6708; (*f*) L. M. Mirica, M. Vance, D. J. Rudd, B. Hedman, K. O. Hodgson, E. I. Solomon and T. D. P. Stack, *J. Am. Chem. Soc.*, 2002, **124**, 9332.

3 *Inter alia:* (*a*) H. Getlicherman, C. Giessner-Prettre and J. Maddaluno, *J. Phys. Chem.*, 1996, **100**, 6819; (*b*) T. Lind, P. E. M. Siegbahn and R. H. Crabtree, *J. Phys. Chem. B*, 1999, **103**, 1193; (*c*) C. J. Cramer and Y. Pak, *Theor. Chem. Acc.*, 2001, **105**, 477; (*d*) P. E. M. Siegbahn and M. Wirstam, *J. Am. Chem. Soc.*, 2001, **123**, 11 819; (*e*) P. E. M. Siegbahn, *J. Biol. Inorg. Chem.*, in press.

4 (*a*) K. D. Karlin, D. -H. Lee, H. V. Obias and K. J. Humphreys, *Pure Appl. Chem.*, 1998, **70**, 855; (*b*) N. N. Murthy, M. Mahroof-Tahir and K. D. Karlin, *Inorg. Chem.*, 2001, **40**, 628.

5 (*a*) L. Casella, E. Monzani, M. Gullotti, D. Cavagino, G. Cerina, L. Santagostini and R. Ugo, *Inorg. Chem.*, 1996, **35**, 7516; (*b*) E. Monzani, L. Quinti, A. Perotti, L. Casella, M. Gullotti, L. Randaccio, S. Geremia, G. Nardin, P. Faleschini and G. Tabbì, *Inorg. Chem.*, 1998, **37**, 553; (*c*) L. Santagostini, M. Gullotti, E. Monzani, L. Casella, R. Dillinger and F. Tuczek, *Chem.-Eur. J.*, 2000, **6**, 519.

6 (*a*) T. Klabunde, C. Eicken, J. C. Sacchetini and B. Krebs, *Nat. Struct. Biol.*, 1998, **5**, 1084; (*b*) C. Gerdemann, C. Eicken and B. Krebs, *Acc. Chem. Res.*, 2002, **35**, 183.

7 J. F. Maddaluno and K. F. Faull, *Experientia*, 1988, **44**, 885.

8 J. S. Conrad, S. R. Dawso, E. R. Hubbard and K. G. Strothkamp, *Biochemistry*, 1994, **33**, 5739.

9 O. Toussaint and K. Lerch, *Biochemistry*, 1987, **26**, 8567.

10 (*a*) M. P. Jackman, A. Hajnal and K. Lerch, *Biochem. J.*, 1991, **274**, 707; (*b*) L. Bubacco, J. Salgado, A. W. J. W. Tepper, E. Vijgenbomm and G. W. Canters, *FEBS Lett.*, 1999, **442**, 215.

11 Jaguar 4.1: Schrödinger Inc., Portland OR, USA, 2000.

12 B3LYP: (*a*) J. P. Perdew, *Phys. Rev. B: Condens. Matter*, 1986, **33**, 8822; (*b*) A. D. J. Becke, *Chem. Phys.*, 1989, **90**, 5648; (*c*) C. Lee, W. Yang and R. G. Parr, *Phys. Rev. B: Condens. Matter*, 1988, **37**, 785

13 (*a*) N. Gresh, C. Policar and C. Giessner-Prettre, *J. Phys. Chem. A*, 2002, **106**, 5660; (*b*) C. Giessner-Prettre and J. Maddaluno, unpublished results.

14 The ELF analysis has been performed using the all-electron DZVP2 basis set: N. Godbout, D. R. Salahub, J. Andzelm and E. Wimmer, *Can J. Chem.*, 1992, **70**, 560 , on a ''standard'' arrangement of the $Cu_2(ImH)_6$ complex (geometry detailled in ref. 3a).

15 (*a*) B. Silvi and A. Savin, *Nature (London)*, 1994, **371**, 683; (*b*) A. Savin, B. Silvi and F. Colonna, *Can. J. Chem.*, 1996, **74**, 1088. For a review, see: ; (*c*) A. Savin, R. Nesper, S. Wengert and T. F. Fässler, *Angew. Chem., Int. Ed. Eng.*, 1997, **36**, 1808.

16 P. S. Bagus and C. W. Bauschlicher, Jr., *J. Chem. Phys.*, 1984, **80**, 4378.

17 J. -P. Piquemal, A. Marquez and C. Giessner-Prettre, in preparation.

18 L. Casella, personal communication.

19 (*a*) K. A. Magnus, H. Ton-That and J. E. Carpenter, in *Bioinorganic Chemistry of Copper*, eds. K. D. Karlin and Z. Tieklár, Chapman and Hall, New York, 1993, pp. 143–150; (*b*) K. A. Magnus, B. Hazes, H. Ton-That, C. Bonaventura and J. Bonaventura, *Proteins*, 1994, **19**, 302; (*c*) K. A. Magnus, H. Ton-That and J. E. Carpenter, *Chem. Rev.*, 1994, **94**, 727.

20 F. Himo, L. Noodleman, M. R. A. Blomberg and P. E. M. Siegbahn, *J. Phys. Chem. A*, 2002, **106**, 8757.

21 (*a*) S. Itoh, M. Taki, H. Nakao, P. L. Holland, W. B. Tolman, L. Que, Jr. and S. Fukuzumi, *Angew. Chem., Int. Ed.*, 2000, **39**, 398; (*b*) V. Fusi, A. Llobet, J. Mahia, M. Micheloni, P. Paoli, X. Ribas and P. Rossi, *Eur. J. Inorg. Chem.*, 2002, 987.

22 However, Flock and Pierloot have shown that in the case of NH_3 ligands, the order of stability of the two binding modes is reversed when changing from the DFT computational procedure to CASPT2: M. Flock and K. Pierloot, *J. Phys. Chem. A*, 1999, **103**, 95.