
Quantum chemical studies of redox-active enzymes

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In applications on mechanisms for metalloenzymes, the hybrid density functional method B3LYP has been used in most cases. The present knowledge about the accuracy of this method on transition metal complexes is described. In comparison to *ab initio* methods like CASPT2 and CCSD(T), B3LYP has generally been shown to perform quite well. However, there is one exception and this is for the type of copper dimer which is found in enzymes like hemocyanin, catechol oxidase and tyrosinase. Large deviations have been found between CASPT2 and B3LYP and also between B3LYP and experiments on model complexes. This situation is carefully investigated. The accuracy of B3LYP for the activation of O₂ in enzymes is also analyzed by comparisons to experiments. For the barrier of O–O bond cleavage B3LYP appears to behave quite satisfactorily, while for the binding of O₂ to the metal there are deviations compared to experiment. The question whether this is due to the B3LYP method or to the chemical models used is addressed.

I. Introduction

The study of metalloenzymes using quantum chemical methods of high accuracy is a relatively new field.¹ During the past five years a quite good understanding has been reached concerning the methods and models to be used for these systems. For systems containing transition metals, hybrid density functional theory (mainly B3LYP) has been used in most applications. Since there are no standard benchmark tests including transition metals, the accuracy of the DFT method used is still not satisfactorily known. One way to check this accuracy could be to compare to highly accurate methods like CCSD(T) and CASPT2. One problem in this context is that these methods can only be used on rather small models. Another one is that these *ab initio* methods have their own problems for transition metal complexes and these are not very well understood at the moment. A few such comparisons have still been made and these will be briefly discussed here. Another way of evaluating the accuracy is to compare optimized structures with those available experimentally. However, apart from the by now well known fact that most methods do quite well on structures, there is not much additional information to be expected from this type of comparison. Comparisons made have generally shown that for one system one method is slightly better while for another system the opposite occurs. Therefore, a better way of evaluating the accuracy of a method for redox-active enzymes is to compare to measured rates for the enzyme which are usually available. Very accurately measured rates are not needed for this comparison since the accuracy for a method like B3LYP is not expected to be better than a few kcal mol⁻¹ for a barrier, which corresponds to an uncertainty for the rate of a factor 100–1000. There are still several problems in this context. First, transition states have to be located for relatively large model systems. Second, when a barrier has been obtained the error compared to experiment could come from other sources

than the method. For example, some important part of the enzyme has perhaps been left out of the chemical model. To address this problem, the chemical models can be tested by adding or subtracting certain features of the models and check for the stability of the results. During the past five years a relatively large number of systems have been studied and compared to experiments in this way and some patterns start to emerge. Some of these comparisons will be discussed in the present paper.

II. Computational details

The calculations discussed in the present paper were usually performed in three steps. For each structure considered, a full geometry optimization was performed using the hybrid density functional B3LYP method.² For open shell systems unrestricted DFT was used. In this first step, standard double zeta basis sets were used for all light elements. For metals non-relativistic effective core potentials (ECP's) were used.³ The valence basis set used in connection with these ECP's is essentially of double zeta quality (the *lacvp* or LANL2DZ basis sets). When Hessians were computed the same basis set was used.

In the second step, the B3LYP energy was evaluated at the optimized geometries using a larger basis set, either the *lacv3p*** basis or the LANL2DZ basis extended with additional functions. These basis sets are essentially similar in accuracy with triple zeta quality and with polarization functions on each atom. In some cases also other basis sets were used in these single-point calculations. Whenever these results are discussed below the basis sets used will be explicitly mentioned.

In the third step, the surrounding environment was treated with self-consistent reaction field methods. Unless otherwise stated, the dielectric constant of the homogeneous dielectric medium was set equal to 4.0, usually found to be representative for enzymes,⁴ and the probe radius was set to 1.40 Å corresponding to the water molecule. The same basis set as for the geometry optimizations was used. The calculations were carried out using either the Jaguar program⁵ or the Gaussian program.⁶

III. Results and discussion

There are numerous tests of the B3LYP method for molecules containing first and second row atoms. For example, several DFT methods have been tested for a standard benchmark test containing 55 molecules.⁷ A few general conclusions can be drawn from these comparisons. For the atomization energies, the B3LYP method is clearly superior to other DFT methods with an average deviation to experiments of only 2.20 kcal mol⁻¹, almost as good as the corresponding result of 1.16 kcal mol⁻¹ obtained for the G2 method,⁸ which is one of the most accurate *ab initio* methods available. For the geometries of the G2 benchmark test, all DFT methods give quite accurate results, perhaps slightly more accurate for the hybrid methods. A few failures of B3LYP are also well known, such as the incorrect dissociation of H₂⁺ and the too low barrier for H₃. Two comments can be made in this context. First, the error for H₃ is about 5 kcal mol⁻¹ which is large compared to the error found with accurate *ab initio* methods, but is not very large compared to errors that must be expected for barriers for the large model systems discussed here. With an average error of 3 kcal mol⁻¹, some individual errors of 5 kcal mol⁻¹ must be expected. The second comment concerns H₂⁺. Several small molecules show the same problem but there seems to be a general tendency that this type of self-interaction error becomes smaller, or less common, for larger systems. At least, so far no case of this self-interaction instability has been reported for an enzyme problem or for a saturated metal complex. There are also reports of B3LYP failures for infinite systems, like a chain of acetylenes⁹ and for the LiF crystal.¹⁰

Due to the lack of accurate experimental values, much less is known about the accuracy of DFT methods for transition metal complexes. For the tests that have been made, the errors are largest for the smallest systems where the splitting between the atomic states directly enter the bond-strengths. The errors of these splittings can be up to 10 kcal mol⁻¹ using B3LYP. For the bond-strengths of MCH₃⁺ and MCH₂⁺, the largest errors compared to experiments are therefore of this size. As the systems get more saturated, the most preferred d-occupation of the metal becomes

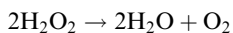
stabilized and the errors in the ligand bond-lengths level out. For the final M–CO bond strength in saturated $M(\text{CO})_n$ complexes the errors are typically within the experimental error bars of a few kcal mol^{-1} , while for the first M–CO bond strength the error can be 10 kcal mol^{-1} or more. Fortunately, the final bond strength is usually the only one that matters for enzyme mechanisms.

In order to make reliable comparisons between model calculations and experiments, the chemical models used first have to be trusted. During the past five years there has been a very intense study and development of different models and some confidence in the models has now been reached. Some tests of the chemical models will be mentioned in the first subsection below.

Since most of the major errors reported for B3LYP on transition metal complexes occur for small unsaturated systems, they are not very relevant in actual mechanistic problems for enzymes. However, there is one notable exception where a large error is found for a normal saturated metal complex. This occurs for the energy difference between the peroxide and bis- μ -oxo states of copper dimers present in hemocyanin, tyrosinase, and catechol oxidase. This problem will be discussed in the second subsection below. The third subsection will deal with the cleavage and binding of O_2 since there are by now many examples of this reaction in enzymes studied using B3LYP. The comparisons to experiments lead to conclusions which will be discussed in the final subsection.

a. Tests of chemical models

To test the model used to study an enzyme mechanism is a natural part of every mechanistic investigation. Therefore, over the past 5–7 years a large amount of experience has been gathered. The most systematic and thorough investigation has been done for manganese catalase, where different models were tested for the entire catalytic cycle.¹¹ Manganese catalase catalyzes the following reaction,



and the purpose is to protect cells from oxidative damage. The active site has a manganese dimer with two histidines and three glutamates, see Fig. 1. The reaction sequence involves many typical enzymatic steps, like O–H bond cleavage, O–O bond cleavage, ligand exchange *etc.* The chemical model used included only this complex and the substrate without any second shell residues. The first questions asked concerning the model were of technical nature. Different basis sets were used both for the optimization of the geometries and for the evaluation of the final energies. It was very conclusively shown, in line with a wealth of earlier experience, that polarisation functions are not needed in the B3LYP geometry optimization. Adding polarization gave such small changes of the final energetics that the effects were essentially within the roundoff errors of the calculation. For the final energies, on the other hand, the results are much more sensitive to the basis set size. It was shown that at least triple zeta quality including polarization is needed. This is normally sufficient but notable effects can sometimes be obtained by going to even larger basis sets. The second set of questions concerning the models were of more chemical nature. It was first shown that in the case of manganese catalase, the effects of adding dielectric effects were small, on the order of a few kcal mol^{-1} , but for high accuracy they are needed. This has been the most common situation for the enzymes studied so far, but other cases exist where charge separations are larger. If the effects are small, the absolute errors coming from the arbitrariness of the choice of the dielectric constant, are

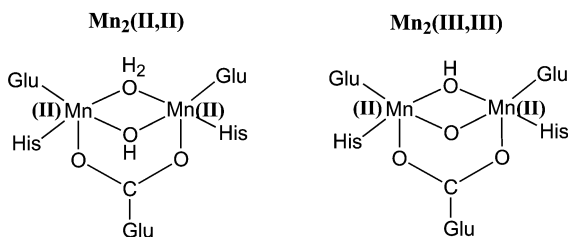


Fig. 1 The Mn-dimer found at the active site in manganese catalase. Two oxidation states have been observed, $\text{Mn}_2(\text{II},\text{II})$ and $\text{Mn}_2(\text{III},\text{III})$.

obviously also quite small. The choice of ligand models were also tested and it was found that glutamates can equally well be modeled by the small formates as the larger acetates. Histidines can almost equally well be modeled by ammonias, even if this seldom is done, as by imidazoles. The only problem is that the ammonias sometimes become involved in artificial hydrogen bondings which can not be formed with histidines. Therefore, histidines are best modeled by imidazoles, which avoids this problem, and this has been done in the applications discussed here. In summary, a confidence of the chemical models has been reached so that the errors coming from the model can be reasonably well controlled, provided that the model is carefully tested from case to case.

b. Copper dimer complexes

As discussed above, most of the known cases of failure for B3LYP are not really relevant for enzyme studies. However, there is one exception that stands out and which has been studied extensively, and this is the copper dimer system in Fig. 2. This complex appears in many enzymes such as hemocyanin, tyrosinase and catechol oxidase. It is thus not at all as exotic as the other systems with known B3LYP failures. In particular, the energy difference between the peroxide and the bis- μ -oxo structure, with an intact and a cleaved O–O bond of O₂, respectively, has been calculated using different methods. Most notably, Flock and Pierloot used the CASPT2 method with large basis sets and active spaces and obtained the bis- μ -oxo structure preferred by 12.7 kcal mol⁻¹. In contrast, using the same basis set with B3LYP they found the peroxide more stable by as much as 19.9 kcal mol⁻¹,¹² indicating a huge error for B3LYP. In line with this result, model complexes experimentally known to have bis- μ -oxo structures have been studied using B3LYP by Lam *et al.*,¹³ who instead got the peroxide more favored and by as much as 21.5 kcal mol⁻¹. Both these studies thus indicate huge errors for B3LYP on an ordinary saturated metal complex found in many enzymes.

In order to obtain more information about this problematic case, the above problem has been studied here again using B3LYP. First, a similar type of system as in the CASPT2 study was studied with a similar result as obtained in that study at the B3LYP level. Second, the system studied by Lam *et al.* was studied. For exactly the same system that gave a large B3LYP preference for the peroxide of 21.5 kcal mol⁻¹, the present B3LYP calculation gave a much reduced preference for the peroxide of only 0.9 kcal mol⁻¹. Extensions of the basis set indicates a further favoring of the bis- μ -oxo structure. What is the large difference between these seemingly similar studies? Lam *et al.* used a somewhat larger basis set, a double zeta basis with d-functions on oxygen, to optimize the geometries but did not extend this basis for the energy evaluation in the final points, as has been generally done before.^{1,14,15} This basis set with only d-functions on oxygen, turns out to be imbalanced and therefore artificially favors the peroxide by about 10 kcal mol⁻¹. The second problem in the Lam *et al.* study was that the optimal structure was not found, leading to another error of 10 kcal mol⁻¹ also favoring the peroxide. The renewed investigation of this system thus leads to B3LYP results in quite good agreement with experiments and the previous conclusion concerning the inadequacy of B3LYP has to be revised.

However, the large disagreement between CASPT2 and B3LYP found in the study by Flock and Pierloot still remains. In this context it is interesting to note that the X-ray structure of hemocyanin^{16,17} actually indicates that the peroxide should be more stable since it is the state observed, which thus agrees with the B3LYP and not with the CASPT2 result. But is it possible that CASPT2

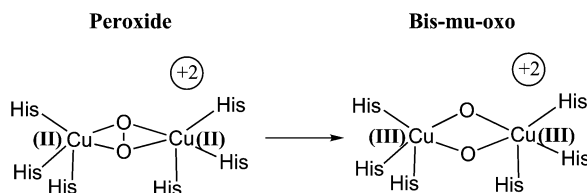


Fig. 2 The copper dimer complex found at the active site in hemocyanin, catechol oxidase and probably also in tyrosinase.

could have an error of nearly 20 kcal mol⁻¹? A few recent very important papers by Pierloot^{18,19} may provide an answer to this question. For CrF₆ an early CASPT2 calculation with 10 active electrons in 10 orbitals gave a preference for the octahedral compared to the trigonal structure of 49.9 kcal mol⁻¹, while CCSD(T) gave only a preference of 14.2 kcal mol⁻¹²⁰ and B3LYP one of 16.9 kcal mol⁻¹. The result at the CASSCF level was a preference for the trigonal structure of 30.4 kcal mol⁻¹. The CCSD(T) result is probably close to correct. The origin of the CASPT2 error was analyzed and was found to be due to the presence of strong covalency in the ligand bonding. To describe this type of bonding properly a much larger active space would be required. Pierloot suggests that 36 electrons in 23 active orbitals would be required which is completely out of reach. This is not the only problematic CASPT2 case, Fe(n)-porphine is another one. In large basis set CASPT2 calculations with 14 electrons in 13 active orbitals the quintet state was found lower than the triplet by 19.6 kcal mol⁻¹.²¹ In contrast, a B3LYP calculation performed here gives a favoring of the triplet by a few kcal mol⁻¹, which is in agreement with experimental interpretations. A simple rule of thumb could be that whenever the CASPT2 result is qualitatively different from the underlying CASSCF result there are reasons to be careful with the CASPT2 result, which can then in some cases be in error by as much as 20 kcal mol⁻¹. The present copper dimer is such a case with a CASSCF preference for the peroxide of 23.2 kcal mol⁻¹ and a CASPT2 preference for the bis- μ -oxo state of 12.7 kcal mol⁻¹, a difference of 35.9 kcal mol⁻¹. Ideally a much larger active space should be chosen than the one used with 12 electrons in 14 orbitals, but this is impossible.

c. Dioxygen activation and binding

Dioxygen cleavage is by now probably the most well studied reaction using B3LYP for enzymes.^{1,14,15,22} This is not because it is the simplest one to study. On the contrary, this is perhaps the most complicated reaction from an electronic structure point of view, with an elaborate flow of electrons and protons needed to achieve the bond breakage. Many states of different spins and with different oxidation states are furthermore usually involved. Some of these studies have therefore been going on for five years and are still in progress. If this is not the simplest reaction, it is certainly one of the most interesting ones, being a key step in many different enzymes. The enzymes where this step has been studied by B3LYP include cytochrome c oxidase,²³ manganese catalase,²⁴ methane mono-oxygenase,²⁵ ribonucleotide reductase,²⁶ intra- and extra-diol dioxygenases,²⁷ tyrosinase,²⁸ amino acid hydroxylases,²⁹ isopenicillin n synthase,³⁰ and cytochrome c peroxidase.³¹ For catechol oxidase the present calculations gave an approximate transition state as shown in Fig. 3, which is quite typical, showing a large coupling between O–O bond stretching and proton motion. A few main patterns emerge from these studies which will be briefly described here.

The first pattern from the model calculations is that B3LYP in general works very well. There is no case of failure as far as can be judged at present. A problem in this context is that the rate of this individual step is seldom known experimentally. What is known is the overall rate, but it can generally be assumed that O–O bond cleavage should either be, or be very close to, rate-limiting implying that the rate of this step is the same as that of the overall reaction. If this is assumed, the calculated B3LYP barrier is in all cases studied so far within 5 kcal mol⁻¹ of the correct barrier. Furthermore, there is no case where the barrier has been computed to be too low but it has always been found slightly too high. Two comments should be made in this context. First, from comparisons for small systems, like H₃ mentioned above, it has generally been concluded that the barriers computed using B3LYP are too low. This shows one of the difficulties of extrapolating results obtained from small systems to realistic cases. The problem of drawing conclusions from small systems was mentioned also above for unsaturated metal complexes which usually give larger errors than saturated ones. The second comment made on the fact that the computed barriers have been found slightly too high is that this means that the error of the method may in reality be smaller. While an error in the chemical model giving a too low barrier can generally be ruled out, this is not the case when a too high barrier has been obtained. Since the enzymes are optimized to minimize the barrier for the rate-limiting step, some effect decreasing the barrier is possible coming from a residue outside the direct active site which may have been left out of the chemical model.

A second pattern from the model calculations is that the binding of O₂ to the metal, which always occurs prior to O–O bond activation, is found to be very small at the B3LYP level. In fact, when entropy effects are added the binding is quite often found to be strongly endergonic by up to

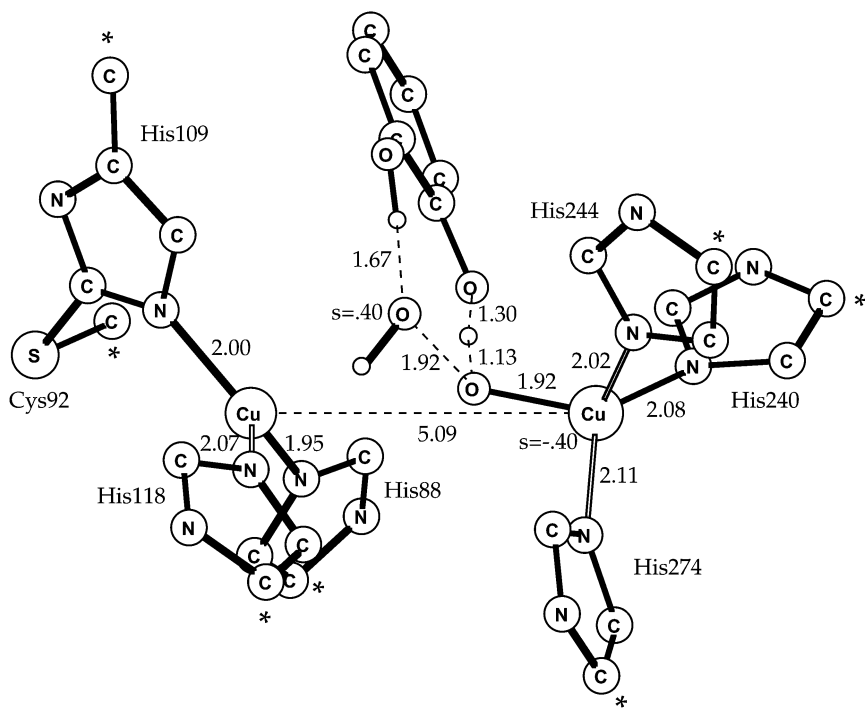


Fig. 3 Approximate transition state for O–O bond cleavage in catechol oxidase. Unimportant hydrogen atoms are not shown. Distances/Å and spins larger than 0.10 are marked. Positions marked by * were frozen from the X-ray structure.

10 kcal mol⁻¹, which does not appear reasonable. A comparison was therefore made to the CCSD(T) method using a very large basis set, for the simple model of Cu–O₂⁺. The CCSD(T) result is a binding between Cu⁺ and O₂ of 10.0 kcal mol⁻¹. The corresponding result for B3LYP is 13.7 kcal mol⁻¹, which does not indicate a severe problem. A large B3LYP error for the O₂ binding to the real complexes in the enzyme can, of course, still not be excluded. A recent QM/MM study of hemerythrin has suggested another explanation for the underestimation of the O₂ binding energies.³² This study gave the surprising result that van der Waals effects from the protein contributes by as much as 6.3 kcal mol⁻¹ to the binding energy of O₂ to iron. Adding also other enzyme effects this essentially cancels the entropy loss when O₂ becomes bound. This result is so surprising that more case studies are required to establish it, but it does fit with the general underestimations of O₂ binding found in B3LYP studies where van der Waals effects from the enzyme have not been accounted for.

IV. Conclusions

The present situation concerning the accuracy of calculations on mechanisms of redox-active enzymes has been briefly discussed with new examples. The hybrid DFT functional B3LYP has after 5–7 years of experience been established as the most useful tool for these systems. An important question in this context concerns, of course, the inherent accuracy of B3LYP for this difficult class of problems. While the accuracy of different methods can be very well determined for molecules containing first and second row atoms by comparisons to well defined benchmarks, the situation for transition metal complexes still relies on a more general experience in comparison to experiments. With the lack of detailed and accurate experimental energies one possibility is to compare to results from methods like CASPT2 and CCSD(T), which in principle can be more accurate. One difficulty in this context is that these methods are limited in size to rather small

models, which can be quite different from the systems of actual interest. It is by now a general experience that small unsaturated transition metal complexes in many ways represent a more difficult class of problems than larger complexes. For example, for unsaturated complexes the metal d-occupation commonly changes rapidly, leading to large correlation changes, while for larger systems this rarely happens. When somewhat larger systems are treated, compromises often have to be made in the CASPT2 and CCSD(T) calculations. It has been demonstrated here for the case of copper dimers, that such compromises can lead to quite wrong conclusions about the accuracy of B3LYP in comparisons to methods like CASPT2. For CCSD(T) there are major problems with even weak near-degeneracies which can lead to large errors, and for larger systems there are also practical problems to use the basis sets required to reach high accuracy. A double zeta plus polarization basis set is hardly enough to obtain high accuracy using CCSD(T).³³ A problem often pointed out when hybrid functionals, like B3LYP, are used is their artificial preference for high-spin states.³⁴ Even though the fraction of exact exchange in B3LYP is not optimally determined by the fitting procedure,² the 20% used at present is in practice not far from optimal for transition metal complexes. There are suggestions that this fraction should be reduced to 15%,³⁴ but to change the present fraction requires a lot more experience. Much more experience is also needed to better define the accuracy of B3LYP for general transition metal complexes, and more information in this context will undoubtedly become available by critical testing the next decade.

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