Is the Bis-μ-Oxo Cu_2(III,III) State an Intermediate in Tyrosinase?

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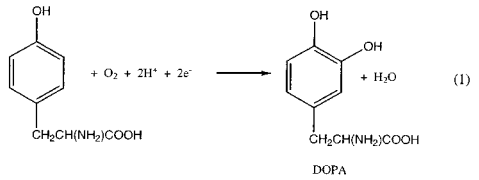
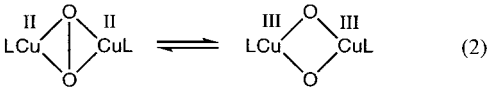
Tyrosinase catalyzes the aerobic oxidation of phenols to *o*-diphenols, the cresolase reaction, eq 1,



(1)

and the subsequent two-electron oxidation of *o*-diphenols to *o*-quinones.[1,2] With tyrosine as substrate, the intermediate reaction product is DOPA, and the final product is a quinone which polymerizes to the pigment melanin. No crystal structure is yet available for tyrosinase. However, chemical and spectroscopic studies of hemocyanin and tyrosinase have shown that the active sites of these enzymes must be extremely similar,[1a] and for hemocyanin there are X-ray structures for both an oxy and a deoxy form.[3,4] In hemocyanin the active site contains a copper dimer complex, where each copper has three terminal histidine ligands. In the deoxy form the oxidation states of the coppers are Cu(I), and in the oxy form, where there is a μ-η^2-:η^2 bridging peroxide, they are both Cu(II) which are antiferromagnetically coupled to an EPR-invisible singlet state. In the following it will be assumed that the active complex is the same one for tyrosinase.

A critical question in the tyrosinase mechanism is how the O−O bond of O_2 is cleaved by the copper dimer complex. In this context there has been important work on synthetic copper dimer model compounds mainly by the research groups of Kitajima,[5] Karlin,[6] and Tolman.[7] For example, Tolman and co-workers found that a μ-η^2-:η^2 peroxide Cu_2(II,II) dimer can be reversibly converted by O−O bond cleavage to a bis-μ-oxo Cu_2-(III,III) dimer (eq 2, L = triazacyclononane)



(2)

under very mild conditions. It has furthermore been shown that

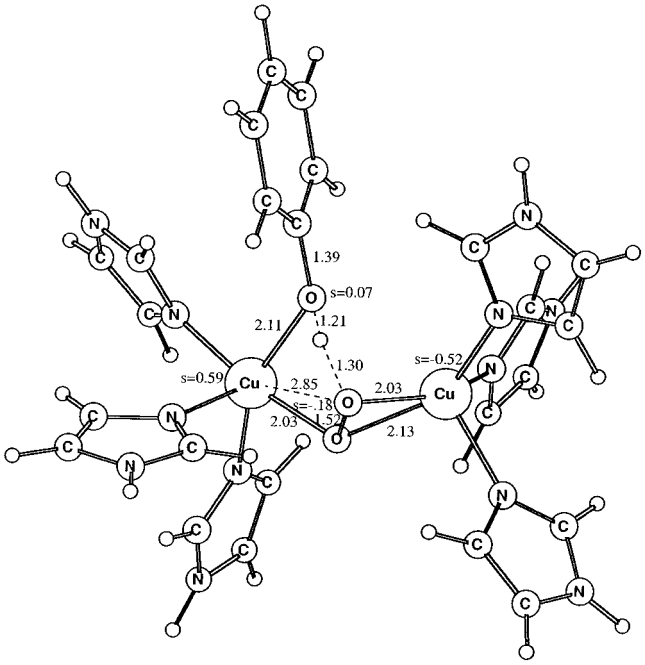


Figure 1. Optimized transition state for O−H activation of tyrosine by the copper-dimer complex of tyrosinase. Distances in Å and spins larger than 0.05 are marked.

the bis-μ-oxo Cu_2(III,III) state is capable of activating C−H bonds in intramolecular N-dealkylation reactions. This model work therefore suggests that this state could be a reactive intermediate in the tyrosinase mechanism even though no such species has been directly observed in any enzyme so far. As an alternative to the formation of the Cu_2(III,III) state, it has been suggested that the O−O bond scission and substrate activation could occur simultaneously (i.e., the peroxo unit attacks the arene ring directly).[1a]

In the present study the question whether the Cu_2(III,III) state is a low-lying intermediate in the tyrosinase catalytic cycle is tested using the hybrid density functional method B3LYP.[8] For this purpose reaction 2 is studied using a model (with charge +2) where all six histidine ligands are modeled by imidazoles (no tyrosine substrate is included in these calculations). In subsequent calculations the tyrosine substrate, modeled by phenol, is added, leading to a model with 71 atoms, see Figure 1. The calculations follow the same strategy as used in several similar recent studies.[9] The geometry is first fully optimized using a medium size double-ζ basis set (in the present case the lacvp basis). In the optimized geometry a single-point calculation is made using a larger polarized basis set (in the present case the lacv3p** basis). The protein environment is accounted for by dielectric cavity methods (dielectric constant 4) in the optimized geometries. Zero-point vibrational effects should be small and were not evaluated due to the size of the system. The computer program Jaguar was used,[10] which is very efficient for large systems. Reaction 2 as a model for tyrosinase has been studied previously by theoretical methods,[11] but not using models of the present size.

The optimized geometry for the Cu_2(II,II) peroxy state shows a reasonable general agreement with the X-ray structure of the oxy-form of hemocyanin with a bent Cu−O_2(midp.)-Cu angle and a Cu−Cu distance of 3.5 Å (exp. 3.6 Å). The Cu−O_2−Cu

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part of the Cu_2(III,III) bis-μ-oxo state is planar, and the Cu−Cu distance has shortened to 2.9 Å. The energy difference between these structures turns out to be as high as 23.3 kcal/mol in favor of the peroxo state. The bis-μ-oxo state has a closed shell wave function while for the peroxo state, the ferromagnetic triplet and antiferromagnetic singlet states are found to be almost degenerate (within 1 kcal/mol) at the present level of geometry optimization. In a recent study it has been shown that if spin-projection is used in the geometry optimization the splitting may increase by up to 4 kcal/mol,[12] which would increase the presently calculated energy difference in favor of the peroxo state even further. Since the total charge state of the model could affect the energy difference between the states, and this charge state is not completely obvious, the calculations were repeated with a neutral model where two imidazoles (one on each copper) were deprotonated. This hardly affected the energy difference between the two states, only by 1.4 kcal/mol in favor of the bis-μ-oxo state. Finally, the energy of the triplet state of the bis-μ-oxo form was calculated and found to be 8.6 kcal/mol higher than the closed-shell singlet state. The conclusions from these calculations is that the Cu_2(III,III) bis-μ-oxo state is a very unlikely intermediate in tyrosinase. The question is why this state forms in the model complexes. The answer most likely is that the strain in the tridentate ligands used strongly favors the bis-μ-oxo form, as clearly demonstrated in model calculations by Bérces[11c] (see Table 5 in that work). The strong strain induced by these ligands is very unlikely to have a correspondence in the normally very weak strain found in proteins, as shown by model work of Ryde et al.[13] Repeatedly, quite accurate structures are obtained by the present methods without accounting for the (minor) strain of the enzyme, and this is true also for the present system from comparisons to the X-ray structure of hemocyanin, see above. The effects on the energetics (mechanism) should be even smaller since the energies are quite insensitive to details of the geometries. Another important result from the study by Bérces, relevant for the present work, is that it was shown that for the tridentate model complexes DFT gives very good agreement with the experimental picture with a fast and reversible interconversion between the peroxo and bis-μ-oxo isomers. There is thus no reason to suspect that DFT should not work well also for the present case. Since Cu_2(III,III) is considered an unlikely intermediate based on an endothermicity as high as 23.3 kcal/mol, a very high accuracy of DFT is not required for drawing this conclusion. It should be added that in our previous study on tyrosinase where ammonia, or formamide, ligands were used,[11e] the Cu_2(III,III) state was found to be much lower in energy and therefore more accessible. The conclusion is that the results using the previous model are not reliable on this point. This is not due to an electronic difference between ammonia and imidazole as ligands[14] but instead due to the formation of a number of artificial hydrogen bonds.

After the main conclusion of the present communication was reached, that the Cu_2(III,III) state is a very unlikely intermediate in tyrosinase, a preliminary investigation was made to find an alternative first step in the catalytic cycle. In these first investigations a protonated tyrosine substrate was assumed present at the same stage as the bridging peroxo form of the copper complex is formed. A simple possibility then is that the bridging peroxide abstracts a proton from tyrosine, which would be similar to a recently suggested alternative pathway for some substrates in P-450[15] and MMO.[16] The optimized transition state for this reaction in tyrosinase is shown in Figure 1. This structure was reached in a stepwise optimization in which the phenol O−H distance was frozen at different values and all other degrees of freedom were optimized. In this way the energy passes smoothly over a maximum. The fact that an explicit Hessian was not used, since it was too time-consuming to be evaluated, should therefore have only a minor effect, estimated to be less than 1 kcal/mol on the barrier. The reaction can be described as an initial binding of tyrosine to an empty coordination site on one of the copper centers. In this reactant structure the peroxide has shifted from the initial μ-η^2-:η^2 coordination without tyrosine to a μ-η^1-:η^2 coordination in which one of the coppers only forms a bond to one of the oxygens of the peroxide. A short hydrogen bond of only 1.47 Å is formed between the tyrosine OH group and the peroxide in this reactant Michaelis complex. The spin-distribution remains largely unchanged with about 0.5 spins on each copper, but with a minor increase of 0.2 on the peroxide. This spin-distribution remains the same at the transition state, as shown in Figure 1, where the proton has moved to a position between tyrosine and the peroxide. In the product complex the resulting tyrosinate becomes bridging with equal distances to the copper centers of 2.02 Å. The hydroperoxide is also bridging in a similar way with equal distances between the unprotonated oxygen and the coppers of 2.03 Å. The protonated oxygen of the hydroperoxide has significantly longer distances to the coppers. The spins on the coppers have increased slightly to 0.6 for this structure. For all structures along this reaction pathway, the ferromagnetic and antiferromagnetic coupling of the copper spins have very similar energies.

The calculated barrier using the large basis set and including dielectric effects is 7.9 kcal/mol. This is a quite feasible barrier since the rate-determining step in the cresolase reaction is 10^3 s^{-1},[17] corresponding to a barrier of 13 kcal/mol. The reaction is endothermic by 5.2 kcal/mol, indicating that the product tyrosinate should not be directly observable. The dielectric effects included in these values are very small with less than 1 kcal/mol, which means that the results are very insensitive to the choice of dielectric constant. The effects of going to the large basis set are more pronounced. For the small basis set the barrier is only 3.5 kcal/mol and the endothermicity 1.6 kcal/mol. Still, a reoptimization of the geometry using the larger basis set is expected to have only a minor effect on the final energies of less than 1 kcal/mol, based on previous experience from careful investigations.[14]

The above suggestion for the first step of the tyrosinase cycle is clearly only one of several possibilities. Work is in progress on other alternatives and on the further steps of the mechanism. One possible next step is a cleavage of the O−O bond in the bridging protonated peroxide ligand. The advantage of cleaving the O−O bond at this stage is that the Cu_2(III,III) state can be avoided by instead creating a tyrosyl and a bridging oxygen radical, which means maintaining a Cu_2(II,II) state after the O−O bond is cleaved. The tyrosyl radical state is indeed found to be much lower in energy than the Cu_2(III,III) state and is only 5 kcal/mol less stable than the starting peroxo reactant. Another possibility presently tested is that tyrosine becomes deprotonated by a ligand other than O_2. In both of these scenarios tyrosinate is formed prior to the cleavage of the O−O bond, as suggested to be the case by recent experiments.[18] While these alternatives remain open, the present main result that the Cu_2(III,III) state is a very unlikely intermediate in tyrosinase should remain valid.

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