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Relative Acidities of Ortho-Substituted Phenols, as Models for Modified Tyrosines in Proteins

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The effects of a variety of ortho-substituents (CH_3, OH, OCH_3, SH, SCH_3, NH_2, NO_2, F, Cl, CN, and imidazole) on the acidity of phenol are investigated using hybrid density functional theory. Substitutions are made at the ortho-position to model modified tyrosine residues found in enzymes. Although the experimental trends are reproduced, the calculations tend to exaggerate the substituent effects. It is shown that the cysteine cross-link to tyrosine, present in the radical enzyme galactose oxidase, has a small effect on the pK_a of the residue. The histidine cross-link present in cytochrome *c* oxidase, on the other hand, will contribute more to lower the pK_a. Comparing the substituent effects on the O−H bond strengths and the acidities, no simple correlation is found between the two.

Introduction

Two enzymes are now known to have active site tyrosines covalently cross-linked to other amino acids at the ortho-position to the phenolic oxygen.

Galactose oxidase (GO) is a mononuclear copper enzyme that catalyzes the two-electron oxidation of a large number of primary alcohols to their corresponding aldehydes, coupled with the reduction of dioxygen to hydrogen peroxide.[1] An interesting feature that was revealed in the X-ray structure is that the active site tyrosyl radical (Tyr272) is connected to a neighboring cysteine (Cys228) through a thioether bond;[2] see Figure 1A. It has been suggested that this thioether bond is in part responsible for the 0.5−0.6 V lowering of the oxidation potential of this species compared to tyrosyls in other enzymes.[3] Whittaker et al. measured an increase in acidity of 0.7 pH units for *o*-(methylthio)cresol relative to parent cresol (10.2 to 9.5).[4]

The second enzyme that is known to have a modified tyrosine at the active site is cytochrome *c* oxidase (CcO). This enzyme is a key part of the respiratory chain and catalyzes the reduction of O_2 to water.[5] The crystal structures showed that the active site tyrosine (Tyr244) and histidine (His240) residues are covalently linked to each other (Figure 1B).[6] Babcock and co-workers have suggested, by analogy to photosystem II, that the Tyr-His moiety acts as a hydrogen atom donor during the O−O bond cleavage.[7] Recently, van der Donk and co-workers measured the pK_a difference between *p*-cresol and 2-imidazol-1-yl-4-methylphenol.[8] They found that the imidazole substitution lowers the pK_a by 1.6 units (10.2 vs 8.6).

To develop more understanding for the perturbations that these substitutions introduce to the properties of the active site tyrosines, and ultimately gain deeper insight into their catalytic roles, it is thus important to characterize these molecules further. We have previously studied the effects of a number of substituents, at the ortho-position from the phenolic oxygen, on the O−H bond strength of phenol and the hyperfine properties of the resulting phenoxyl radical.[9]

In that study, it was demonstrated that for ortho-substitutions, in contrast to meta and para, intramolecular hydrogen bonding between the phenolic oxygen and the substituent is quite important for the bond energies. The hydrogens of the oxygen and the substituents can point in different directions, giving rise to several distinct well-defined minima (see Figure 2). The energy differences can be very large, as in the extreme case of nitro-substitution, where the difference between having the phenolic hydrogen pointing toward NO_2 or away from it is as large as 10.7 kcal/mol in the gas phase. The effects on the other substituents are smaller, but still substantial, up to 5 kcal/mol. For this reason it was concluded that experimental substituent effects on the O−Me bond in anisole cannot be directly transferred to the O−H bond in the phenol molecule.

It was in particular shown that the biologically interesting substitutions, cysteine and histidine (modeled by methylthiol and imidazole, respectively), gave small lowering of the bond dissociation energy (BDE) relative to unsubstituted phenol. The changes were −1.7 and −1.0 kcal/mol for SCH_3 and imidazole substituents, respectively. Furthermore, the hyperfine parameters were not significantly perturbed by these particular substitutions.

Our objective in the present work is to study the effects of ortho-substitutions on the acidity of phenol. The substitutions considered are the same as in the previous study: CH_3, OH, OCH_3, SH, SCH_3, NH_2, F, Cl, CN, NO_2, and imidazole. The theoretical method we have used (also the same as before) is the Hartree−Fock/density functional theory (HF/DFT) hybrid method B3LYP.[10]

Ab initio pK_a calculations are generally governed by two factors, the underlying gas-phase calculations and the solvation calculations, with the accuracy of the former tending to be more critical for the overall accuracy.[11] In this context, the methodology employed in the present study, B3LYP/6-311+G(2d,2p) with self-consistent isodensity polarized continuum model (SCI-PCM) solvation calculations, can be considered as adequate.

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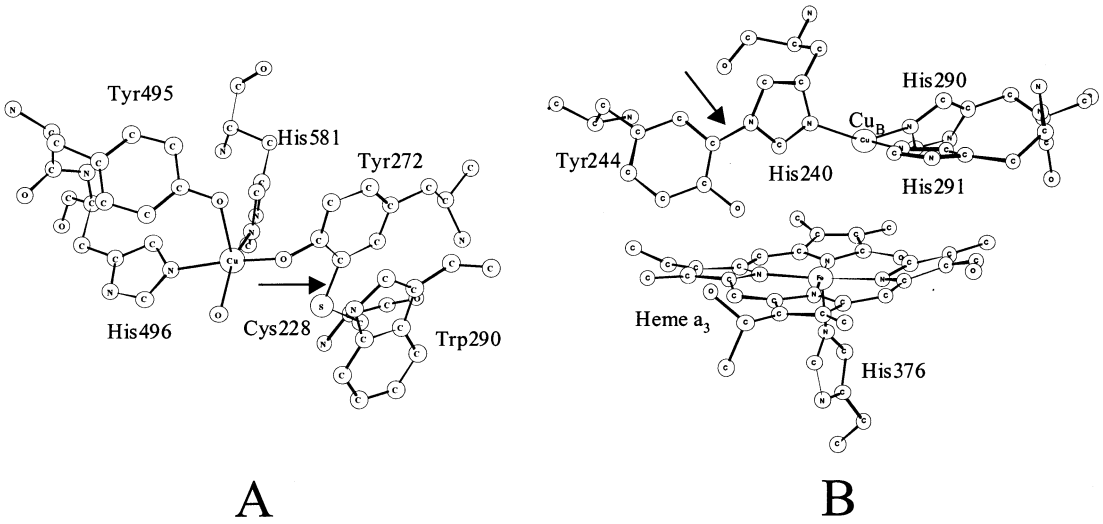

Figure 1. Active site structures of (A) galactose oxidase and (B) cytochrome *c* oxidase. Arrows indicate cross-links.

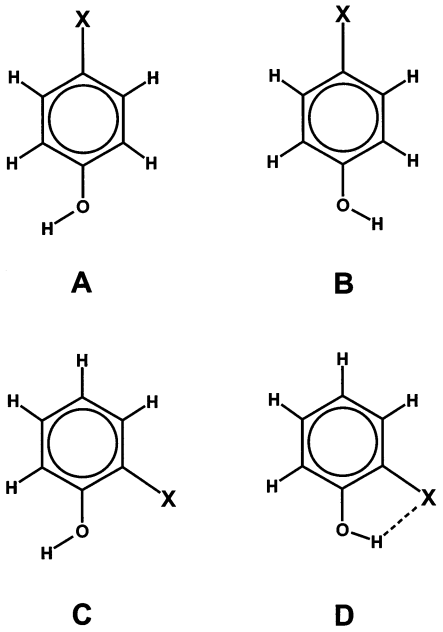

Figure 2. Difference between para- and ortho-substitution in terms of intramolecular hydrogen bonding. The two isoenergetic structures A and B for para-substitution become energetically distinct in the case of ortho-substitution, C and D.

To our knowledge, the issue of relative acidities of ortho-substituted phenols has not been addressed theoretically before. The only theoretical study directly relevant to our investigation is the one by Schüürmann.[12] The conductor-like screening model (COSMO) in DFT as well as semiempirical continuum-solvation models were tested against a number of chlorophenol acidities measured by Saito et al.[13] The relative pK_a's for this set of molecules were well reproduced, in particular, the 1.5 units drop (from 9.8 to 8.3) in the *o*-chlorophenol. However, the COSMO-DFT method was not found to be sufficiently accurate to predict absolute pK_a values. The reason for this was believed to be the atomic radii used in the solvation calculations, and also uncertainties in the underlying gas-phase calculations.[12]

Theoretical Methods

All the calculations reported in the present study were carried out using the density functional theory (DFT) functional B3LYP,[10] as implemented in the Gaussian94 program package.[14] Geometries were optimized with the double-ζ plus polarization basis set 6-31G(d,p). On the basis of these geometries, more accurate energies were calculated using the large basis set, 6-311+G(2d,2p). This is a triple-ζ basis set with one diffuse function and two polarization functions on each atom.

Zero-point energies (ZPE) were assumed to be constant between the unsubstituted and substituted systems and were therefore not calculated. This assumption was validated in our previous study,[9] and also by other investigators.[15]

To calculate the pK_a values, the restricted B3LYP scheme was used for both the neutral and anionic species, because both are closed shell systems. In the previous work on the BDE, the unrestricted B3LYP scheme was used to calculate the radical species.[9]

Solvent effects were calculated as single point calculations with the large basis set 6-311+G(2d,2p), using the self-consistent isodensity polarized continuum model (SCI-PCM)[16] implemented in the Gaussian program. We also tested optimizing the geometries under PCM, but the effects were very small, on the order of 0.1 kcal/mol. The dielectric constants used were $\epsilon = 80$ and $\epsilon = 4$, to model water solution and the protein environment, respectively. Initially, the solvent effects were calculated with the smaller basis set, 6-31G(d,p) and added to the energetics of the large basis set. However, it was observed that these two effects, i.e., the basis set effect and the solvent effect, are not additive. The errors made are in the range 3−5 kcal/mol for absolute proton affinities and up to 1 kcal/mol for relative ones.

The pK_a values are calculated using the relation

$$\mathrm{p}K_a = \frac{\Delta G}{\ln(10)RT}$$

where R is the gas constant and T is the absolute temperature.

For a system in solution, the thermodynamic cycle depicted in Scheme 1 gives

$$\Delta G_{sol} = \Delta G_{gas} + \Delta G_3 + \Delta G_4 - \Delta G_1 - \Delta G_2$$

Solvent effects on water and hydronium ion (ΔG_2 and ΔG_4) are poorly described with the PCM method. However, these are constant terms and cancel in the subtraction when calculating the relative pK_a's between substituted and unsubstituted phenols (ΔpK_a).

SCHEME 1: Thermodynamic Cycle for Calculation of pK_a Value of Phenols

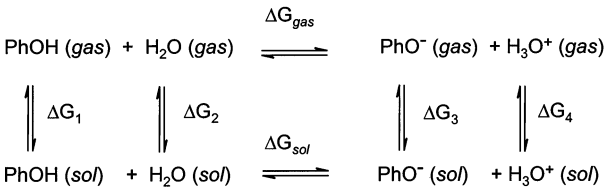

TABLE 1: B3LYP/6-311+G(2d,2p)//B3LYP/6-31G(d,p) Calculated Relative pK_a's of Ortho-Substituted Phenols[a]

substituent	calc ΔBDE[b] (kcal/mol) (gas phase)	calc ΔpK_a (gas phase)	calc ΔpK_a ($\epsilon = 4$)	calc ΔpK_a ($\epsilon = 80$)	expt ΔpK_a
H	0.0	0.0	0.0	0.0	0.0
CH_3	−2.2	−0.2	+0.7	+0.8	+0.3[c]
OH	−9.7	−7.2	−5.6	−4.8	
OCH_3	−1.9	+3.6	+3.3	+2.4	
SH[d] (phenol)	−4.6	−4.9	−4.1	−3.7	
SH[e] (thiol)		−15.4	−13.1	−12.4	
SCH_3	−1.7	−0.7	+0.2	−0.5	−0.7[f]
NH_2	−11.6	−1.4	−0.1	+0.3	
F	+1.1	−2.3	−2.8	−3.2	
Cl	+2.0	−4.0	−3.5	−3.6	−1.5[g]
CN	+4.2	−11.2	−9.2	−8.7	
NO_2	+13.2	−7.8	−6.7	−6.7	−2.7[c]
imidazole	−1.0	−9.4	−4.9	−3.5	−1.6[h]

[a] Experimental relative pK_a's and relative gas phase O−H bond dissociation energies (kcal/mol) are given for comparison. [b] From ref 9. [c] Reference 21. [d] With the S−H bond frozen. [e] pK_a values are for thiol proton. [f] Reference 4. [g] Reference 13. [h] Reference 8.

Effectively, the relative pK_a between substituted, Ph(X)OH, and unsubstituted, PhOH, phenols is calculated using the relations ($T = 298$ K, and ΔG given in kcal/mol)

$$\Delta pK_a = 0.73\Delta G$$

$$\Delta G = \{E[\mathrm{Ph(X)OH}] - E[\mathrm{Ph(X)O^-}]\} - \{E[\mathrm{PhOH}] - E[\mathrm{PhO^-}]\}$$

Results and Discussion

Before discussing the results, it is important to make a technical point. As pointed out in the Introduction, the hydrogens of the oxygen and the substituents can point in different directions, giving rise to several energy minima. These hydrogen bonding effects were discussed at greater length in the previous study on relative BDE's.[9] For the present study it is sufficient to note that it was carefully verified that the correct lowest minima were used in the calculation of the pK_a values.

Calculated relative acidities along with the previously obtained relative bond dissociation energies are presented in Table 1. The relative pK_a's are calculated in the gas phase as well as in solution, where the SCI-PCM technique was used with $\epsilon = 80$ and $\epsilon = 4$ to model the water solution and protein interior surroundings, respectively.

Generally, the results can be analyzed in terms of two counteracting effects. The first is the electron withdrawing power of the substituents, which decreases the pK_a relative to unsubstituted phenol by polarizing the O−H bond and also by delocalizing the negative charge and thereby stabilizing the resulting anion. The second effect is the stabilization of the neutral parent species compared to the anion caused by the above-mentioned intramolecular hydrogen bonding. This results in an increased pK_a relative to unsubstituted phenol. The relative magnitudes of these two effects determine the acidity of the species.

We first note that, though reproducing the general trends satisfactorily, the calculations tend to exaggerate the effects of the substituents. Chlorine substitution, for instance, is calculated to reduce the pK_a by 3.6 units, whereas the experiments give a decrease of 1.5 units.[13] Similarly, nitro-substitution results experimentally in a decrease of 2.7 units, whereas the calculations give a decrease of as much as 6.7 units. The calculated 3.2 and 8.7 unit drops in pK_a due to F- and CN-substitution are therefore also believed to be overestimated. Other theoretical studies give similar exaggerations for these kinds of substituents.[11]

As seen from Table 1, there is no obvious correlation between the substituent effects on the O−H BDE and pK_a. A naive intuitive picture would be that if it is easier to remove a proton, it should be easier to remove a hydrogen atom. It is, however, easily seen from the Bordwell kind of relation[17]

$$\mathrm{BDE_{HA}} = 1.37\mathrm{p}K_{\mathrm{HA}} + 23.1E_{\mathrm{ox}}(\mathrm{A^-}) + C$$

that this is not necessarily the case, because the redox potential, $E_{ox}(A^-)$, of the species is also involved. In our calculations presented in Table 1, there are some cases that are illustrative.

One extreme case is aminophenol, for which the BDE is lowered by 11.6 kcal/mol relative to unsubstituted phenol, whereas the pK_a is rather unaffected. The large drop in the BDE was rationalized in the previous study in terms of resonance structures introduced by the NH_2-substitution, stabilizing the radical species relative to the parent molecule.[9] These kinds of radical resonances do not appear in the phenolate form of the molecule.

On the other end of the spectrum there is the nitro-substituted phenol. Although the BDE is as much as 13.2 kcal/mol higher than in unsubstituted phenol, the pK_a is lowered by as much as 7.8 pK_a units in the gas phase (−6.7 in solution and −2.7 experimentally). The nitro-substituent is a strong electron-withdrawing group and is hence expected to lower the pK_a. For BDE, this property is less critical, and the intramolecular hydrogen bonding in the parent molecule results in the BDE increase.

As is the case for the relative BDE's, there is a large difference between the effects of hydroxy- and methoxy-substitutions. This is mainly due to the stabilizing intramolecular hydrogen bonding that exists in *o*-hydroxyphenolate but is lacking in *o*-methoxyphenolate. *o*-Hydroxyphenol is accordingly about 11, 9, and 7 pK_a units more acidic than *o*-methoxyphenol, in the gas phase, $\epsilon = 4$, and $\epsilon = 80$, respectively.

Let us now discuss the biologically interesting substitutions. Modeling the cysteine cross-link in galactose oxidase by a simple SH group will not correctly describe the actual chemistry of this species. The reason is that the thiol proton is more acidic than the phenolic (calculated difference in $\epsilon = 80$ is ca. 9 pK_a units). In fact, when the geometry of the SH-substituted phenolate is optimized, the proton moves over automatically during the course of the optimization from the sulfur atom to the oxygen. This calculated difference is likely to be an overestimation, in line with the discussion above, and also considering that the experimental pK_a difference between unsubstituted phenol and unsubstituted thiophenol is only ca. 3.4 units. Using methylthiol (SCH_3) as a more correct model of cysteine, a lowering of the pK_a by only 0.5 pK_a units is obtained in water solution, in excellent agreement with the

experimental finding of a 0.7 unit decrease.[4] This is yet another indication that the cysteine cross-link in galactose oxidase has little effect on the electronic structure and hence the chemistry of the tyrosine residue. Previously, we also calculated small effects on the O−H bond strength,[9] and on the spin distribution and hyperfine properties.[18,19]

The imidazole cross-link is calculated to lower the pK_a by 3.5 units in $\epsilon = 80$. This is an overestimation compared to the measured −1.6 decrease[8] and is in line with the above-mentioned observation that the effects of strong electron-withdrawing substitutions are generally overestimated.

In contrast to the cysteine substitution, the effect of imidazole substitution on the phenol acidity seems to be highly dependent on the surrounding environment. The pK_a difference relative to unsubstituted phenol decreases from −9.4 to −4.9 to −3.5 units, for gas phase, protein, and water solution, respectively.

This large dielectric dependency can be rationalized in the following way. Due to steric effects, the phenyl and imidazole rings cannot be coplanar. In the X-ray structure the angle between them is ca. 47°, whereas in the calculations, the neutral species has an angle of ca. 59° and the anionic species an angle of ca. 27°. This will result in low charge delocalization onto the imidazole ring in the phenolate form of the species (Mulliken population calculations predict ca. 0.13). The solvent sensitivity originates hence from the fact that the almost charge-neutral imidazole ring excludes the solvent from the charged phenolate oxygen, making the imidazole-substituted anion therefore less and less stable compared to the unsubstituted case as the dielectric constant increases.

With respect to the biological activity in cytochrome *c* oxidase, it has been argued[8] that the decrease in the pK_a of the tyrosine residue caused by the histidine cross-link facilitates proton delivery to the binuclear center in the reductive half-reaction, which occurs via a proton channel involving the tyrosine residue. On the other hand, the formation of a tyrosyl radical in the oxidative part of the catalytic cycle does not seem to be energetically significant.[20] There is therefore no need to decrease the tyrosine OH-bond strength, in agreement with the minor change obtained computationally for the OH-bond strength in the cross-linked compound.[8,9] The present calculations also indicate that the pK_a value of the cross-linked tyrosine can be further lowered by the low dielectric protein environment and by the specific surrounding of the tyrosine residue, whereas previous calculations showed that the OH-bond strength is not significantly affected by the surrounding medium.[9]

Finally, a word about the geometries. The geometries of ortho-substituted phenols and phenolates are almost unaffected by the nature of the substituent. The C−O distance, for instance, varies in the range of 1.35−1.37 Å for the different phenols, and 1.25−1.29 Å for the different phenolates. When going from the phenols to the corresponding phenolates, the only notable geometric change (apart from the obvious shortening of the C−O bond length) is seen for the two C−C bonds closest to C−O, which are elongated by ca. 0.05 Å.

Conclusions

We have in the present paper used density functional theory combined with continuum dielectric solvation calculations to calculate the relative acidities of ortho-substituted phenols. The calculations were motivated by crystallographic studies showing active site tyrosines in the galactose oxidase and cytochrome *c* oxidase enzymes being cross-linked to other amino acids at the ortho-position.

One interesting finding in this study is that the O−H bond dissociation energy and the acidity of the phenol are affected quite differently by the substitutions. As a striking example we have discussed the amino-substituted phenol, where the BDE is lowered by 11.6 kcal/mol relative to unsubstituted phenol, and the pK_a is calculated to actually increase by 0.3 pH units.

With respect to the biologically relevant substitutions, it was found that thioether substitution lowers the pK_a by 0.5 units, in nearly perfect agreement with the 0.7 unit decrease measured experimentally. For the histidine substitution, which was calculated to lower pK_a by 3.5 units (1.6 experimentally) in water solution, it was found that the low dielectric environment of the protein probably causes a larger drop in the pK_a.

In line with other studies, we find that the substituent effects are exaggerated compared to experiments. One likely source of error is the radii used to define the solute−solvent interface. The solvation energy is known to be sensitive to these, and explicit waters might be needed to accurately reproduce the solvation effects.

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