$$R_1^i = \sum_{j \neq i} R_1^{ij} \quad (2)$$

where

$$R_1^{ij} = [\gamma_H^4 h^2][d_{ij}]^{-6}[\tau_c^{ij}] \quad (3)$$

To account for cross-relaxation which occurs during nonselective ^1H T_1 measurements we can write[6]

$$R_1^i(NS) = R_1^i + \sum_{j \neq i} \sigma_{ij} \quad (4)$$

Freeman and co-workers[7] have shown that R_1^i can be measured in the initial rate approximation[7] with the 180° − τ − 90° sequence provided that the 180° pulse is selective (e.g., 10 ms in our experiments) and that, in the extreme narrowing limit,[8] $[R_1^i(NS)/R_1^i] = 1.5$.

In a thoroughly dried and deoxygenated 2×10^{-2} M sample of **1** in Me_2SO-d_6 this ratio was 1.5 for both H-γ_1 and H-γ_2 showing that their relaxation was entirely intramolecular dipole-dipole. Double selective excitation experiments[9] of H-γ_1 and H-γ_2 gave all the experimental data to determine $\sigma_{\gamma_1\gamma_2}$ $\sigma_{\gamma_2\gamma_1}$ from the equations

$$R_1^{\gamma_1}(\gamma_1,\gamma_2) = R_1^{\gamma_1} + \sigma_{\gamma_1\gamma_2}$$

and

$$R_1^{\gamma_2}(\gamma_1,\gamma_2) = R_1^{\gamma_2} + \sigma_{\gamma_2\gamma_1}$$

Here the $R_1^{\gamma}(\gamma_1\gamma_2)$ and R_1^{γ} terms were obtained by double selective and monoselective experiments, respectively. In accordance with theory $\sigma_{\gamma_1\gamma_2} = \sigma_{\gamma_2\gamma_1}$ with a value of 0.290 ± 0.010 s^{-1}. By writing $2\sigma_{\gamma_1\gamma_2} = R_1^{\gamma_1\gamma_2} = \gamma_H^4 h^2 (d_{\gamma_1\gamma_2})^{-6}$. $(\tau_c^{\gamma_1\gamma_2})$ and assuming $d_{\gamma_1\gamma_2} = 1.8$ Å, $\tau_c^{\gamma_1\gamma_2} = (3.4 \pm 0.2) \times 10^{-11}$ s.

The correlation time for the C^{γ}–H vectors was determined from ^{13}C T_1 measurements to be $\tau_c^{CH} = 6.5 \times 10^{-11}$ s, a value appreciably slower than $\tau_c^{\gamma_1\gamma_2}$. Since the ^{13}C T_1 sample had a concentration 40 times that of the −H T_1 sample, this discrepancy is to be attributable to viscosity and/or intermolecular effects. The fact that $\tau_c^{\gamma_1\gamma_2}$ for the ^{13}C T_1 sample was (5.8 ± 0.3) × 10^{-11} s, a value close to $\tau_c^{H\text{-}\gamma} = (6.5 \pm 0.5) \times 10^{-11}$ s, indicates that intermolecular effects are no larger than the experimental error.

(1) By combining nonselective and mono- and diselective excitation T_1 values and known geminal ^1H–^1H distances, τ_c can be calculated. This technique is generally applicable to simple and complex molecules, including amino acid side chains in peptides and proteins. (2) Measurements of τ_c are made at low concentrations and are significantly faster than those at the high concentrations typical of ^{13}C measurements. The two protons involved in these measurements should exhibit first-order coupling. (3) Extention of this method to nongeminal protons is possible, e.g., distances derived from dihedral scalar coupling constants and Karplus curves.

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References and Notes

(1) R. S. Norton and A. Allerhand, *J. Am. Chem. Soc.*, **98**, 1007–1014 (1976).
(2) A. Allerhand and R. A. Komorosky, *J. Am. Chem. Soc.*, **95**, 8228–8231 (1973).
(3) A. Allerhand, D. Doddrell, and R. A. Komorosky, R. A., *J. Chem. Phys.*, **55**, 189–198 (1971).
(4) R. Deslauriers, I. C. P. Smith, and R. Walter, *J. Biol. Chem.*, **249**, 7006–7010 (1974).
(5) A. P. Zens, T. J. Williams, J. C. Wisowaty, R. R. Fisher, R. B. Dunlap, T. A. Bryson, and P. D. Ellis, *J. Am. Chem. Soc.*, **97**, 2850–2857 (1975).
(6) A. Abragam, "The Principles of Nuclear Magnetism", Clarendon Press, Oxford, 1961, p 295–297.
(7) R. Freeman, H. D. Hill, B. L. Tomlinson, and L. D. Hall, *J. Chem. Phys.*, **61**, 4466–4473 (1974).
(8) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect", Academic Press, New York, 1971, p 18–19.
(9) L. D. Hall and H. D. W. Hill, *J. Am. Chem. Soc.*, **98**, 1269–1270 (1976).

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Ultraviolet Resonance Raman Study of Oxytyrosinase. Comparison with Oxyhemocyanins

Sir:

Tyrosinase and hemocyanin are metalloproteins which contain an EPR-nondetectable binuclear copper active site often classified together with the binuclear sites in laccase, ascorbate oxidase, and ceruloplasmin as type 3 copper.[1] Both proteins interact with molecular oxygen: hemocyanin[2] functions as the oxygen carrier for molluscs and arthropods, whereas tyrosinase[3] utilizes oxygen in the hydroxylation of monophenols and the dehydrogenation of *o*-diphenols. Resonance Raman spectroscopy has been used to study the active site structure and mode of oxygen binding for various oxyhemocyanins. This communication reports the results of parallel studies on oxytyrosinase.

Oxytyrosinase is produced by the reaction of either mushroom[4] (*Agaricus bispora*) or *Neurospora crassa*[5,6] tyrosinase with hydrogen peroxide in the presence of oxygen. This protein derivative has an absorption spectrum remarkably similar to that of oxyhemocyanin (hemocyanin, $\epsilon_{345\ nm}$ 20 000 M^{-1} cm^{-1}, ϵ_{570} 1000; oxytyrosinase, ϵ_{345} 18 000, ϵ_{600} 1000) and has been postulated[4] to be a catalytic intermediate in the biological functioning of tyrosinase. Initial resonance Raman studies[7] on oxyhemocyanin (*Cancer magister*) used visible excitation frequencies (457.9–647.1 nm) and revealed an enhanced vibration at 744 cm^{-1} assignable to O–O stretching on the basis of its $^{18}O_2$ isotope shift. The frequency of the O–O stretch indicates that oxygen is bound as peroxide. More recent resonance Raman studies[8] on *Busycon canaliculatum* and *Limulus polyphemus* oxyhemocyanin involved UV excitation (351.1 and 363.8 nm). These spectra exhibit a cluster of bands in the metal–ligand region which have been tentatively assigned as copper–imidazole stretches and a remnant of the 742-cm^{-1} O–O stretch. Parallel resonance Raman studies were undertaken to investigate the increased reactivity of the bound oxygen in oxytyrosinase as compared to that in oxyhemocyanin.

Tyrosinase from *Neurospora crassa* wild-type strain was purified[9] by salt fractionation and a combination of ion-exchange and hydroxylapatite chromatography and stored as microcrystals in 20 mM sodium phosphate buffer, pH 6.8. Raman samples were prepared by dissolving the microcrystals in 20 mM sodium phosphate, 0.5 M sodium chloride, pH 6.8 buffer to a final concentration of 10–20 mg/mL (0.5–1.0 mM in copper). Approximately 30% of the dissolved tyrosinase was in the oxy form. Conversion of the resting protein to oxytyrosinase was accomplished by the addition of a 1.2–1.5 molar excess of H_2O_2 or a 5-fold molar excess of NH_2OH·HCl in the presence of oxygen. Oxytyrosinase containing $^{18}O_2$ (Stohler Isotope Chemicals) was prepared by repeated evacuation and

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Table I. Comparison of the Resonance Raman Spectra of Oxyhemocyanins with That of *Neurospora crassa* Oxytyrosinase[a]

Busycon canaliculatum	*Limulus polyphemus*	*Cancer magister*	*Cancer irroratus*	*Cancer boreolis*	*Neurospora crassa* oxytyrosinase	tentative assignment[b]
119						δ_{NCuN}
170	180 (br)	180 (br)	110–240 (br)	199 (br)	184 (br)	δ_{NCuN}
226	223	218		217	218	ν_{CuN}
267	271 (sh)	262 (sh)				ν_{CuN}
286 (sh)	287	282	288	284	274	ν_{CuN}
315 (sh)	306 (sh)	308 (sh)			296 (sh)	ν_{CuN}
337 (sh)	338	333 (sh)		332	328	ν_{CuN}
749	752	744		748	755	ν_{OO}

[a] In cm^{-1} [b] References 8 and 12, nitrogen atoms from imidazole rings.

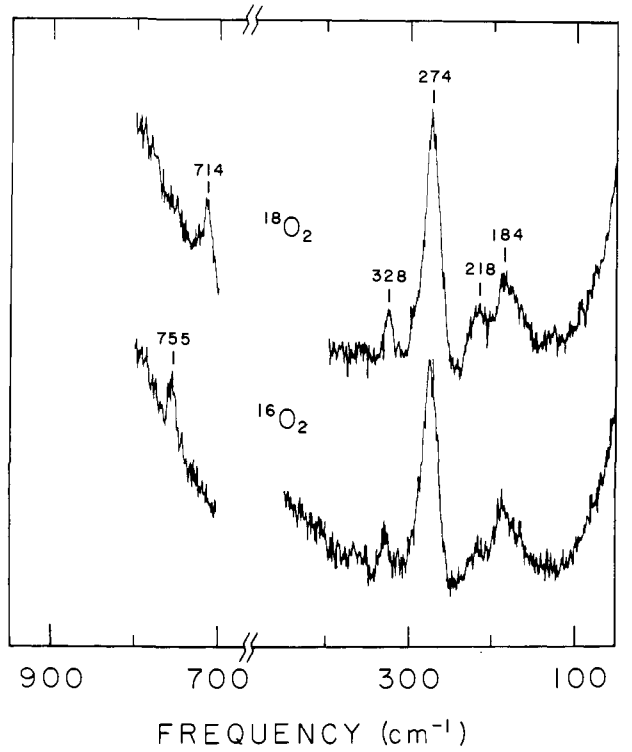

Figure 1. Resonance Raman spectra of (a) $^{16}O_2$ and (b) $^{18}O_2$ *Neurospora crassa* oxytyrosinase, 17.2 mg/mL (0.41 mM in protein, 0.82 mM in copper); 200-mW, 363.8-nm laser excitation; spectral slit width 7 cm^{-1}; 50–450-cm^{-1} region, photon counting for 2 s/cm^{-1}, scale 1500–3000 counts; 700–800-cm^{-1} region, addition of five scans, photon counting for 2 s/cm^{-1}, scale 9500–11500 counts.

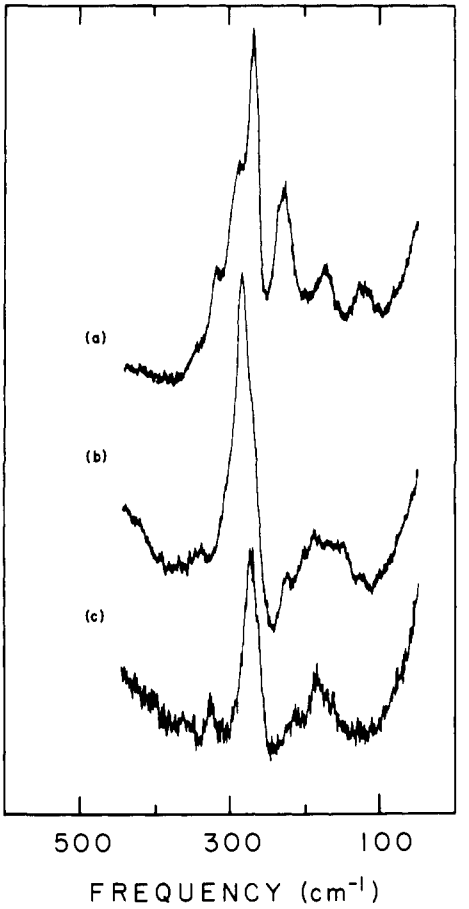

Figure 2. (a) Resonance Raman spectrum of *Busycon canaliculatum* oxyhemocyanin, 1 mM in copper in 0.05 M sodium carbonate buffer, pH 9.8; 100-mW, 363.8-nm laser excitation, spectral slit width 7 cm^{-1}, sensitivity 2000 counts; rise time 3 s; scanning speed 30 cm^{-1}/min. (b) Resonance Raman spectrum of *Limulus polyphemus* oxyhemocyanin, 1.7 mM in copper in 0.05 M sodium carbonate buffer, pH 9.8; 200-mW, 363.8-nm laser excitation, spectral slit width 7 cm^{-1}, sensitivity 2000 counts; rise time 3 s; scanning speed 30 cm^{-1}/min. (c) Resonance Raman spectrum of *Neurospora crassa* oxytyrosinase, instrumental conditions as in Figure 1.

equilibration of resting tyrosinase with argon followed by introduction of $^{18}O_2$ and subsequent addition of hydroxylamine hydrochloride solution. All manipulations of the sample were performed directly in a Raman spinning cell sealed with rubber septa.

Raman spectra were recorded using a Spectra Physics Model 170 Ar^+ laser, a Spex double monochromator, RCA C31034A photomultiplier, and Ortec photon counter. Data were collected and stored by a Data General Nova 2 computer. A small volume (~1 mL) spinning cell was used and a stream of cold nitrogen gas was directed onto the sample during data collection in an effort to prolong sample lifetime. Oxytyrosinase was unstable in the laser beam, turning reddish brown after prolonged exposure (>2 h). Samples were discarded after 1 h and scans were repeated on separate samples to assure reproducibility. This instability precludes any accurate measurement of depolarization ratios or intensity profiles. The low intensity of the 755-cm^{-1} band (vide infra) also discourages any oxytyrosinase experiments with oxygen mixed isotopes such as those done on oxyhemerythrin[10] and oxyhemocyanin.[11]

Laser excitations into the 345-nm absorption band of oxytyrosinase using 363.8- or 351.1-nm lines gives rise to several resonance-enhanced Raman bands between 100 and 350 cm^{-1} and a weaker enhanced band at 755 cm^{-1} (Figure 1a). $^{18}O_2$ oxytyrosinase shows an identical resonance Raman spectrum in the low frequency region, but the 755-cm^{-1} band shifts to 714 cm^{-1} (Figure 1b). A control sample of resting tyrosinase showed only a weak spectrum consistent with the 30% oxytyrosinase present.

In the region between 100 and 350 cm^{-1} (Figure 1) there are at least five bands in oxytyrosinase. The most intense band centered at 274 cm^{-1} probably consists of at least three bands,

but only one shoulder at 296 cm^{-1} is well defined. The 184- and 218-cm^{-1} bands are broad and certainly contain several components. A comparison of the low frequency resonance Raman spectrum of oxytyrosinase with those of *Limulus polyphemus* and *Busycon canaliculatum* oxyhemocyanin is shown in Figure 2. A tabulation of these data and those from two other arthropods is given in Table I. The vibrations enhanced for oxytyrosinase correspond quite closely to those for the oxyhemocyanins, both in energies of vibrations and intensity patterns. These vibrations are most likely metal-ligand stretches and bends but the absence of any shift upon $^{18}O_2$ substitution precludes the assignment of the Cu-O vibrations. Imidazoles have frequently been suggested as protein ligands. Model studies[12] confirm that copper-imidazole vibrations occur at these energies and the observed bands are tentatively assigned as such in Table I.

The energy of the 755-cm^{-1} band indicates that the oxygen is bound as peroxide and the shift to lower frequency of 41 cm^{-1} upon $^{18}O_2$ incorporation (Figure 1) further confirms this assignment as it is close to the calculated shift of 43 cm^{-1} for a pure O-O stretch. Similarly, the 744-cm^{-1} band of *Cancer* oxyhemocyanin shifts by 40 to 704 cm^{-1} upon isotope substitution.[7] The O-O stretch for *Neurospora* tyrosinase is higher than that found for any hemocyanin, but, considering the range of frequencies found for the hemocyanins in Table I, the difference is not significant.

The results of these studies establish that the coordinated oxygen in oxytyrosinase exists as peroxide and strongly suggest that the copper atoms in this derivative are in the divalent state, the lack of an EPR signal being attributable to antiferromagnetic coupling between the Cu(II) ions (cf. ref 13). It has been determined[14] that one oxygen molecule binds per two coppers in *Neurospora* oxytyrosinase, just as in hemocyanin. The correspondence of the peroxide stretch and the metal-ligand vibrations demonstrate that the active sites of oxyhemocyanin and oxytyrosinase are very similar. Since peroxide complexes are known[15] to oxygenate substrates under relatively mild conditions, our results would indicate that the increased reactivity of tyrosinase is due to the details of the substrate-active site interactions rather than the relative activation of the oxygen.

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References and Notes

(1) (a) R. Malkin and B. G. Malmstrom, *Adv. Enzymol.*, **33**, 177 (1970); (b) J. A. Fee, *Struct. Bonding, (Berlin)*, **23**, 1 (1975).
(2) K. E. Van Holde and E. F. J. van Bruggen in "Subunits In Biological Systems," Part A, S. N. Timasheff and G. D. Fasman, Ed., Marcel Dekker, New York, 1971, pp 1-53.
(3) W. H. Vanneste and A. Zuberbuhler in "Molecular Mechanisms of Oxygen Activation", O. Hayaishi, Ed., Academic Press, New York, 1974, pp 374-394.
(4) (a) R. L. Jolley, Jr., L. H. Evans, and H. S. Mason, *Biochem. Biophys. Res. Commun.*, **46**, 878 (1972); (b) R. L. Jolley, Jr., L. H. Evans, N. Makino, and H. S. Mason, *J. Biol. Chem.*, **249**, 335 (1974).
(5) S. Gutteridge and D. Robb, *Eur. J. Biochem.*, **54**, 107 (1975).
(6) K. Lerch, *FEBS Lett.*, **69**, 157 (1976).
(7) (a) J. S. Loehr, T. B. Freedman, and T. M. Loehr, *Biochem. Biophys. Res. Commun.*, **56**, 510 (1975); (b) T. B. Freedman, J. S. Loehr, and T. M. Loehr, *J. Am. Chem. Soc.*, **98**, 2809 (1976).
(8) J. A. Larrabee, T. G. Spiro, N. S. Ferris, W. H. Woodruff, W. A. Maltese, and M. S. Kerr, *J. Am. Chem. Soc.*, **99**, 1979 (1977).
(9) K. Lerch, unpublished work.
(10) D. M. Kurtz, Jr., D. F. Shriver, and I. M. Klotz, *J. Am. Chem. Soc.*, **98**, 5033 (1976).
(11) T. J. Thamann, J. S. Loehr, and T. M. Loehr, *J. Am. Chem. Soc.*, **99**, 4187 (1977).
(12) J. A. Larrabee and T. G. Spiro, *J. Am. Chem. Soc.*, submitted for publication.
(13) R. S. Himmelwright, N. C. Eickman, and E. I. Solomon, *J. Am. Chem. Soc.*, submitted for publication.
(14) K. Lerch, unpublished results.
(15) V. J. Choy and C. J. O'Connor, *Coord. Chem. Rev.*, **9**, 145 (1972/73), and references therein.

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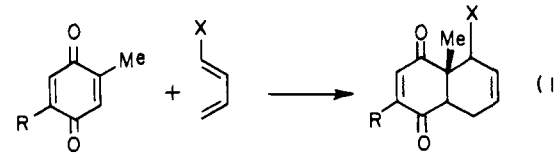
Schizophrenic Substituents: The Origin of Anomalous Substituent Effects on Cycloaddition Regioselectivity

Sir:

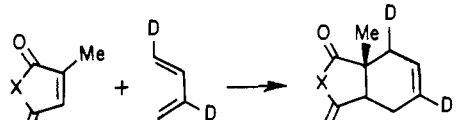
The frontier molecular orbital (FMO) method has proven valuable in the rationalization and prediction of cycloaddition regioselectivity.[1] However, it is surprising that, in some cases, small difference in sizes of terminal FMO coefficients nevertheless lead to high regioselectivity; in a few other cases, predictions of FMO theory are clearly at odds with experiment. We report here model calculations which reveal two important general refinements of the FMO method, and also provide explanations of anomalous regioselectivities observed in a large class of cycloadditions.

Bohlmann and co-workers recently reported several Diels-Alder reactions of substituted benzoquinones (eq 1) and pointed out that the regioselectivities of these Diels-Alder reactions could not be understood on the basis of frontier molecular orbital (FMO) theory.[2] Numerous other reactions, summarized in eq 2 and 3,[3,4] are of a specific type which has not been treated explicitly by frontier MO theory:[1] the electron-deficient partner in the cycloaddition is made unsymmetrical by substitution of a methyl group, which is usually thought of as a monolithic donor group. Our previous generalizations suggest that donors, including methyl, cause the π^* LUMO of an otherwise symmetrical alkene to be polarized in such a fashion that the larger LUMO coefficient will be possessed by the donor-substituted carbon.[1a,5] Bohlmann's HOMO calculations on the quinones used in his studies verify

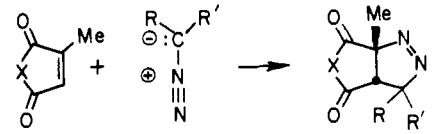


(1)

R = Me or MeO; X = Me, CH_2OAc, CO_2Me



(2)[3]



(3)[4]

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