

Figure 2. Chemical shift of thymine 4 NH vs number of equivalents of added adenine 5 in the presence (O) and absence (\diamond) of 20.0 mM SDS. Line corresponds to the best-fit curve calculated for a 1:1 binding isotherm ($K = 16.09 \text{ M}^{-1}$, $\delta_{\text{unbound}} = 10.682 \text{ ppm}$, $\delta_{\text{bound}} = 11.552 \text{ ppm}$). Titrations were performed on a 500-MHz NMR instrument at $22 \pm 1 \text{ }^\circ\text{C}$. H_2O was used as a reference (δ 4.65).

basis of a reported mean aggregation number of 60 for SDS,⁷ the receptor should comprise about 3 molecules of 4 among 60 molecules of SDS at 20 mM SDS and 1.0 mM 4. In the absence of SDS, ammonium salt 4 does not significantly self-associate in aqueous solution, exhibiting variations in chemical shift of less than 0.01 ppm over a concentration range of 0.5–25 mM.

Receptor 1 binds adenine derivatives in aqueous solution. Binding studies were performed by ^1H NMR titration of a solution of 1.0 mM 4, 20.0 mM SDS, and 1.0 mM AcOH (to reduce the rate of exchange of the thymine NH) in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ with a solution of 200 mM acetyl adenine 5 and 20.0 mM SDS in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$. A ^{133}I pulse sequence⁸ provided water-suppression and permitted monitoring of the thymine NH resonance.⁹ Substantial downfield shifts of the NH resonance occur upon addition of 5 (Figure 2) and are consistent with adenine–thymine base-pairing.¹⁰ In the absence of SDS, smaller upfield shifts occur, indicating that aromatic-stacking interactions predominate.¹¹ Analysis of the micelle data affords an excellent fit to a 1:1 binding model for the association of 4 and 5 and reveals an association constant of 16 M^{-1} .^{12,13}

Our data support a model in which the micelles exclude bulk water from the hydrogen-bonding surface of the thymine group, thus providing a microenvironment suitable for binding.⁴ The binding constant in aqueous SDS (16 M^{-1}) is smaller than that of 3 and 5 in CDCl_3 (37 M^{-1}),¹² suggesting that the thymine group resides in an environment comparable to a polar organic solvent.¹⁴

^1H NMR studies suggest that concentration of 5 inside the micelles may also contribute to binding.¹⁵ Thus, addition of 20 mM SDS to a solution of 5 in 10% $\text{D}_2\text{O}/\text{H}_2\text{O}$ results in small ($\leq 0.02 \text{ ppm}$) shifts in the ^1H NMR spectrum of 5.

In summary, we have found that base-pairing of simple adenine and thymine derivatives occurs in micelles. We anticipate that the incorporation of hydrogen-bonding groups into micelles will prove a general strategy for the design of aqueous molecular receptors.

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Magnetic Circular Dichroism Spectroscopic Definition of the Intermediate Produced in the Reduction of Dioxygen to Water by Native Laccase

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Laccase, a multicopper oxidase, catalyzes the irreversible 4-electron reduction of dioxygen to water. The enzyme contains a blue (type 1, T1) copper and a trinuclear copper cluster comprised of a normal (type 2, T2) copper and a binuclear (type 3, T3) copper center.¹ Intermediates in the reaction of reduced enzyme with dioxygen have been detected in the native enzyme² and in a derivative, T1Hg,³ where the T1 copper is replaced with redox-inactive Hg^{2+} . The intermediate in T1Hg has been shown to be a 2-electron peroxide intermediate, with the T3 oxidized and T2 reduced.⁴ Studies of the intermediate in native laccase have led to proposals that this intermediate is a 3-electron reduced oxygen radical.² Evidence for this includes the rapid reappearance of absorption features at 614 and 330 nm, associated with oxidized T1 and T3, respectively, and lack of a T2 EPR signal.^{2b} In addition, an EPR signal, attributed to the intermediate, is observed at helium temperature which exhibits a low g value and fast relaxation.⁵ ^{17}O line broadening of this signal indicates the direct involvement of oxygen.⁵ To elucidate this intermediate's structure we have employed magnetic circular dichroism (MCD) spectroscopy to probe its electronic properties. The appearance of intense MCD C-terms at 364 and 318 nm provides definitive evidence for the intermediate having significant Cu(II) character. In addition, the T3 site, diamagnetic in the resting enzyme due to antiferromagnetic coupling, is paramagnetic in the intermediate. An alternative description is presented for the electronic structure of this intermediate based on the MCD data. MCD spectroscopy⁶ is found to be a powerful probe of paramagnetic intermediates in reaction mixtures.

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(10) Similar effects were observed with 9-propyladenine. However, the greater basicity of this compound resulted in rapid exchange of the thymine NH proton, permitting observation of only a small fraction of the binding isotherm.

(11) Analysis of this limited range of data affords an association constant of 4.6 M^{-1} .

(12) Association constants were determined by a nonlinear least-squares fitting of NMR titration data to a 1:1 binding isotherm. Values of K , δ_{unbound} , and δ_{bound} were allowed to vary during the fitting procedure.

(13) The large ratio of 5 to 4 rendered accurate observation of the thymine NH resonance difficult at acetyl adenine concentrations greater than ca. 100 mM. For this reason only the first 62% of the binding curve is reported here and analyzed.

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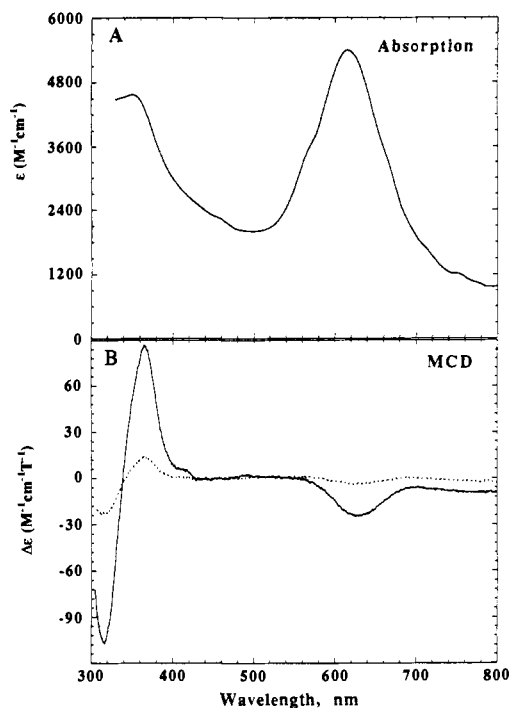


Figure 1. A. Optical absorption spectrum of the oxygen intermediate at 233 K. Samples were prepared in 0.1 M potassium phosphate buffer, pH 7.4, and 50% v/v in ethylene glycol. Protein concentration was ~ 0.1 mM. The spectrum of fully reduced laccase was subtracted. B. Low-temperature MCD of native laccase at 4.2 (—) and 25 K (---) at 5 T. The samples were optical glasses formed in a 0.1 M potassium phosphate pH 7.4 buffer that was 50% v/v in glycerol. Protein concentration was ~ 0.2 mM.

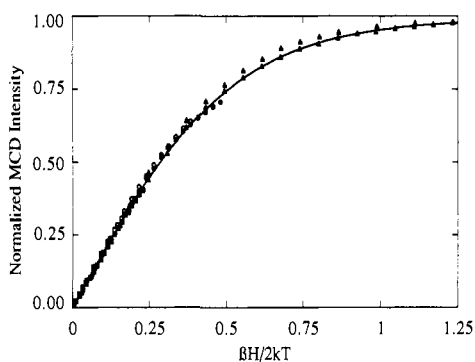


Figure 2. MCD saturation magnetization data of the native laccase intermediate at 1.62 K (Δ), 4.2 K (\circ), and 9 K (\square) for both the 318-nm (open) and 364-nm (solid) features. Protein concentration was ~ 0.4 mM. Magnetic field range was 0–6 T. Solid line is the fit of this data to eq 1.

Intermediate samples were prepared by rapidly quenching the reaction of reduced laccase⁷ and dioxygen in an MCD cell holder with liquid nitrogen.^{8,9} This was accomplished with a double syringe loaded with reduced laccase and oxygen-saturated buffer.¹⁰ The absorption data was obtained using an HP8452A diode array

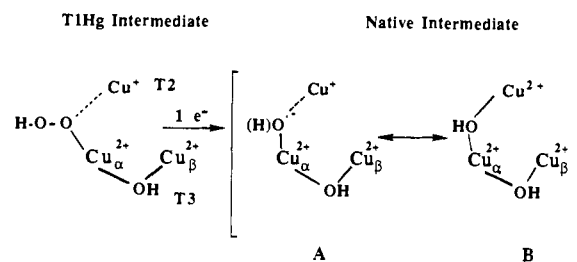
(7) Laccase was isolated and purified from acetone powder (Satio and Co., Osaka, Japan) from the lacquer of the Japanese lacquer tree (*Rhus vernicifera*) as described in (a) Reinhammar, B. *Biochim. Biophys. Acta* 1970, 205, 35–47, and modified according to (b) Spira-Solomon, D. J.; Solomon, E. I. *J. Am. Chem. Soc.* 1987, 109, 6421–6432. Laccase samples routinely had A_{280}/A_{614} ratios in the range 14.5–16.5.

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(9) The lifetime of the intermediate at pH 7.4 for *Rhus vernicifera* laccase is reported to be approximately 20 s. Andréasson, L.-E.; Brändén, R.; Reinhammar, B. *Biochim. Biophys. Acta* 1976, 438, 370–379.

(10) MCD samples were made anaerobic and reduced with 4 equiv of ascorbate. Samples had depolarization ratios of less than 15%.

Scheme 1



spectrometer equipped with an Oxford DN-1704 variable-temperature cryostat.¹¹

Figure 1 presents the absorption and MCD spectra of the oxygen intermediate. A shoulder is observed in the 233 K absorption spectrum at 360 nm in agreement with earlier data.^{2b} The MCD spectra show three transitions at 318, 364, and 610 nm. The 610-nm feature is due to reoxidized T1 copper. These transitions are temperature dependent, indicating that they are MCD C-terms which require these excited states to be associated with a paramagnetic ground state. Saturation magnetization data (Figure 2) for both C-terms do not nest and therefore result from an isolated doublet ground state. These data were fit to the expression for a Kramers doublet,

$$A = A_{\text{sat}} \tanh(g\beta H/2kT) \quad (1)$$

giving an effective g value of 1.94 ± 0.05 for both the 318 and 364 nm bands. The magnetic properties of the MCD C-terms are consistent with the helium EPR signal, thus linking the optical and EPR intermediates.

A pure $S = 1/2$ orbitally nondegenerate ground state will have no MCD C-term intensity as this requires a change in orbital angular momentum for the transition. The spin-orbit coupling constant of an isolated oxygen or hydroxyl radical is too small to mix sufficient orbital angular momentum into the ground state to produce C-term MCD intensity. Note from Figure 1B that the C-term intensity of the intermediate transitions is very high. The MCD to absorption intensity ratio is comparable to that of ligand \rightarrow Cu^{2+} charge-transfer (CT) transitions, where the CT intensity derives from the large spin-orbit coupling of $\text{Cu}(\text{II})$. Thus, the high MCD intensity of the intermediate requires significant Cu^{2+} character in the ground-state wavefunction, which also accounts for the deviation of the g value from 2.0. The 318-nm C-term cannot be associated with the 360-nm absorption feature, but it is close in energy to the 330-nm absorption band of the oxidized T3 site. It therefore appears from the MCD that the T3 binuclear site becomes paramagnetic in the intermediate.

Addition of one electron to the T1Hg peroxide intermediate (Scheme 1) from the additional reduced T1 copper would give the 3-electron reduced oxygen "intermediate" depicted as structure A. However, the EPR hyperfine parameters of the ^{17}O -derived intermediate are $A_{xx} = 5.5$ mT, $A_{yy} = A_{zz} = 0$.^{5b} These give an estimate of the oxygen 2p character in the ground-state wavefunction of only $\sim 33\%$, indicating considerable delocalization onto the T2 copper in agreement with the MCD data.¹² Electronic structure calculations on a tetrahedral $[\text{Cu}(\text{NH}_3)_3\text{O}^-]$ system give 33% oxygen character in the HOMO, which reduces to 10% in going to a tetragonal geometry.¹³ This would lead to the alternative description B in Scheme 1, which is actually a 4-electron reduced oxygen product rather than a 3-electron reduced oxygen intermediate. Structure B, a hydroxide-bridged copper(II) trimer, would have similar antiferromagnetic coupling between both copper(II) pairs and a total spin $= 1/2$ ground state delocalized over the three coppers.^{1c,14} This structure would give MCD

(11) Excess ascorbate was removed from reduced laccase solutions by anaerobic dialysis. The intermediate was generated by reacting reduced laccase with O_2 -saturated 50% v/v ethylene glycol buffer in the cryostat at 233 K. It should be noted that the EPR signal of the intermediate is unaffected by glassing agents.

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activity to the 330-nm T3 transition. Further, the EPR spectrum of this trimer would not resemble that of mononuclear copper(II). Trimer *g* values result from the projection of the individual copper *g* tensors onto the trimer coordinate system with the spin on the central copper opposing the other two spins (see Figure 15 of ref 1c). Noncolinear *g* tensors would give *g* values less than 2.0 and provide an explanation of the helium temperature EPR signal. Structure A, with no interaction between the O^{•-} and a reduced T2 copper, would have the oxygen radical directly overlapping with the T3 Cu_α²⁺, resulting in strong antiferromagnetic coupling between their spins. The magnetic model for such a trimer ($J_{O3\alpha} \gg J_{3\alpha3\beta}$, where $J_{O3\alpha}$ is the O^{•-}-T3 Cu_α²⁺ coupling; ref 1c) would have the spin localized predominantly on the T3 Cu_β²⁺ resulting in an EPR spectrum resembling mononuclear copper(II), which is not observed. These results suggest that the 4-electron reduction of dioxygen to water by laccase may proceed via two, 2-electron steps to a product (structure B in Scheme I) with different spectral properties from the resting enzyme. Subsequent loss of this T2-T3 hydroxide bridge would result in a T2 EPR signal and a diamagnetic T3 binuclear site as found in resting laccase. Experiments are underway to quantitate the nature of the ground-state wavefunction associated with this species (i.e., the relative contributions of structures A and B) in order to provide a complete description of its electronic structure.

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Revised Structure of Bistramide A (Bistratene A): Application of a New Program for the Automated Analysis of 2D INADEQUATE Spectra¹

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Didemnid ascidians (tunicates) are excellent sources of novel biologically active compounds of varied biosynthetic origin;³ *Lissoclinum* spp., in particular, produce peptides⁴ (e.g., *lissoclinum* peptides⁵ and *patellins*⁶), macrolides (e.g., *patellazoles*),⁷ and

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Table I. Bistramide A NMR Data (500 MHz, CDCl₃)

C no.	¹³ C (mult) ^a	¹ H (<i>J</i> _{HH} , hertz) ^b	CC (<i>J</i> _{CC} , hertz) ^c
1	18.33 (q)	1.88 (dd, 6.8, 1.4)	2 (41.7)
2	144.38 (d)	6.85 (dq, 15.7, 6.8)	1 (41.7)
3	132.10 (d)	6.10 (dq, 15.7, 1.5)	4 (53.8)
4	198.86 (s)		3 (53.8), 5 (41.0)
5	45.22 (t)	2.86 (dd, 17.0, 8.9), 2.49 (dd, 17.0, 3.0)	4 (41.0), 6 (41.8)
6	64.84 (d)	4.15 (m)	5 (41.8), 7 (36.5)
7	30.70 (t)	1.61 (m), 1.34 (m)	6 (36.5), 8 (32.5)
8	26.48 (t)	1.58 (m), 1.27 (m)	7 (32.5), 9 (33.1)
9	33.26 (d)	1.89 (m)	8 (33.1), 10 (35.4), 11 (36.2)
10	17.03 (q)	0.82 (d, 7.0)	9 (35.4)
11	74.73 (d)	4.02 (dd, 10.9, 4.6)	9 (36.2), 12 (35.8)
12	32.38 (t)	2.71 (dd, 15.1, 11.7), 2.10 (dd, 14.9, 1.4)	11 (35.8), 13 (51.0)
13	173.36 (s)		12 (51.0)
14	44.61 (t)	3.46 (dt, 14.0, 5.8), 3.19 (dt, 14.0, 5.7)	15 (39.8)
15	73.76 (d)	3.67 (dt, 10.3, 5.1)	14 (39.8), 16 (36.2)
16	43.32 (d)	2.34 (dq, 5.0, 7.0)	15 (36.2), 17 (34.5), 18 (49.0)
17	15.49 (q)	1.21 (d, 7.0)	16 (34.5)
18	175.12 (s)		16 (49.0)
19	39.46 (t)	3.26 (dt, 12.7, 6.6) ^d	20 (36.1)
20	25.80 (t)	1.77 (m), 1.50 (m)	19 (36.1), 21 (35.2)
21	30.41 (t)	1.67 (m), 1.30 (m)	20 (35.2), 22 (40.7)
22	74.22 (d)	3.11 (dt, 9.6, 1.8)	21 (40.7), 23 (36.7)
23	34.82 (d)	1.24 (m)	22 (36.7), 24 (35.4), 25 (33.0)
24	17.94 (q)	0.76 (d, 6.6)	23 (35.4)
25	27.87 (t)	1.52 (m), 1.42 (m)	23 (33.0), 26 (32.7)
26	36.06 (t)	1.57 (m), 1.40 (m)	25 (32.7), 27 (45.2)
27	95.41 (s)		26 (45.2), 28 (45.6)
28	35.44 (t)	1.52 (m), 1.32 (m)	27 (45.6), 29 (33.0)
29	19.17 (t)	1.79 (m), 1.48 (m)	28 (33.0), 30 (32.6)
30	31.30 (t)	1.48 (m), 1.08 (m)	29 (32.6), 31 (36.9)
31	69.02 (d)	3.40 (m)	30 (36.9), 32 (40.5)
32	34.05 (t)	1.33 (m), 1.26 (m)	31 (40.5), 33 (35.5)
33	33.43 (t)	1.33 (m), 1.27 (m)	32 (35.5), 34 (34.5)
34	31.82 (d)	2.31 (m)	33 (34.5), 35 (34.8), 36 (43.7)
35	20.90 (q)	0.90 (d, 6.8)	34 (34.8)
36	131.32 (d)	5.15 (d, 9.2)	34 (43.7)
37	137.16 (s)		38 (43.5), 39 (45.7)
38	11.79 (q)	1.58 (fd, 1.3)	37 (43.5)
39	73.23 (d)	4.16 (m)	37 (45.7), 40 (38.7)
40	21.74 (q)	1.20 (d, 6.3)	39 (38.7)
NH1		7.27 (bt, 5.8)	
NH2		6.93 (bt, 5.5)	
OH1		4.58 (d, 5.3)	
OH2		2.76 (broad) ^e	

^a Determined from DEPT spectrum. ^b Assigned from HMQC, $J_{CH} = 140$ Hz; ¹H-¹H couplings measured from 1D ¹H spectrum. ^c C-C bonds determined from 2D INADEQUATE, $J_{CC} = 40$ Hz. ^d Degenerate methylene protons. ^e Observed in the benzene-d₆ spectrum of bistramide A.

alkaloids (e.g., varamines).⁸ *Lissoclinum bistratum* contains the cytotoxic cyclic peptides, bistratamides A and B, and the macrocyclic ether bistramide A (a.k.a. bistratene A).⁹ This latter compound has demonstrated activity in a variety of systems: cytotoxicity toward MRC5CV1 fibroblasts and T24 bladder carcinoma,¹⁰ P388 murine leukemia, KB, and human endothelial

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