Reassessment of the Active Site Quino-Cofactor Proposed To Occur in the *Aspergillus niger* Amine Oxidase AO-I from the Properties of Model Compounds†

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Received March 6, 2000

ABSTRACT: Quino-cofactors have been found in a wide variety of prokaryotic and eukaryotic organisms. Two variants have, thus far, been demonstrated to derive from tyrosine precursors: these are the 2,4,5-trihydroxyphenylalanine quinone (topa quinone or TPQ) [Janes, S. M., et al. (1990) *Science 248*, 98] and an *o*-quinone analogue containing the side chain of a lysine residue (lysyltyrosine quinone or LTQ) [Wang, S. Z., et al. (1996) *Science 273*, 1078]. Additionally, a third variant of the family of tyrosine-derived cofactors has been reported to exist in an *Aspergillus niger* amine oxidase AO-I. This was described as an *o*-quinone cross-linked to the side chain of a glutamate residue [Frebort, I. (1996) *Biochim. Biophys. Acta 1295*, 59]. We have synthesized model compounds related to the proposed structure. Characterization of the redox properties for the model compound and spectral properties of its 4-nitrophenylhydrazine derivative lead us to conclude that the cofactor in *A. niger* amine oxidase AO-I has been misidentified. A TPQ carboxylate ester is considered an unlikely candidate for a biologically functional quino-cofactor.

Topa quinone (2,4,5-trihydroxyphenylalanine quinone or TPQ)[1] is a covalently bound, active site cofactor in a wide variety of copper-containing amine oxidases (structure **1**, Figure 1) (*1*−*3*). Model compounds for this cofactor show that it is capable of the catalytic oxidation of primary amines to aldehydes (*4*−*8*), analogous to the reaction carried out by the copper amine oxidases (*9*). A variant of TPQ, in which the ϵ-amino group of a lysine side chain replaces the 2-keto group in TPQ, has been documented in lysyl oxidase from bovine aorta (*10*). This is designated lysyltyrosine quinone or LTQ (structure **2**, Figure 1).

The biogenesis of TPQ has been shown to be an autocatalytic process that requires solely the addition of dioxygen and copper to a precursor form of protein (*11*, *12*). Proposed biogenetic schemes invoke the oxidative production of dopa quinone from a tyrosine precursor, followed by the nucleophilic addition of water (or hydroxide ion) to the 2-position of the dopa quinone ring, which after oxidation yields TPQ (Scheme 1) (*3*, *13*, *14*). While little is known about the biogenesis of LTQ, an analogous reaction involving the addition of a lysine side chain to the dopa quinone precursor is considered likely. These pathways for cofactor production raise the possibility of additional quino-cofactors that differ with regard to the nature of the substituent or amino acid side chain that has undergone covalent addition to a dopa quinone intermediate (cf. Scheme 1) (*15*).

In this context, Frebort and co-workers have presented evidence for a variant of TPQ in which the 2-position is formally acylated by the γ-carboxyl group of a glutamic acid

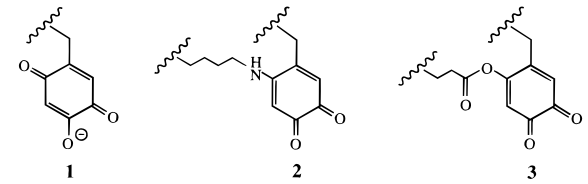
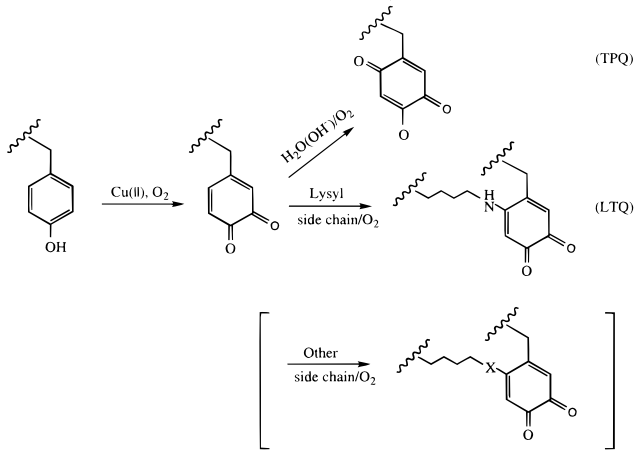


FIGURE 1: Structures for TPQ (**1**), LTQ (**2**), and the proposed cofactor in AO-I (**3**).

Scheme 1: Postulated Pathway for TPQ Biogenesis Involving the Intermediacy of Dopa Quinone[a]



[a] Nucleophilic attack by a protein side chain on the dopa quinone ring may be the pathway for production of LTQ, as well as other, as yet unidentified, quino-cofactors.

† This work was supported by NIH Grant GM 39296 (J.P.K.) and NIH Grant GM 34468 (J.S.-L.).

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[1] Abbreviations: TPQ, 2,4,5-trihydroxyphenylalanine quinone; LTQ, lysyltyrosine quinone; SCE, saturated calomel electrode.

10.1021/bi000507m CCC: $19.00
Published on Web 06/01/2000

residue (structure **3**, Figure 1) (*16*, *17*). In this paper we report the synthesis and characterization of a model compound for structure **3**, for comparison to the spectral properties of the native *Aspergillus niger* amine oxidase AO-I and its derivatives. The properties presented herein argue that the cofactor structure at the enzyme active site is most likely TPQ rather than a glutamic acid cross-linked derivative. From the properties of the model compounds described, we consider it highly unlikely that quino-cofactors will be found that have undergone cross-linking to carboxylic (glutamic or aspartic) acid side chains.

EXPERIMENTAL PROCEDURES

^{1}H NMR and ^{13}C NMR were performed on Bruker AM-400 MH_3 and AM-500 MH_3 spectrophotometers. UV−vis absorbance data were obtained on a HP8450A diode array spectrophotometer equipped with a thermostated cell holder at 25 ± 0.2 °C (path length of 1 cm). Mass spectra were obtained on a VG 70-SE or a VG ZAB 2-EQ instrument. Cyclic voltammetry was performed as previously described (*4*) in pH 7.60 potassium phosphate/potassium chloride buffer. The final buffer was 0.10 M in chloride and 0.20 M in phosphate. Midpoint potential values (E_m) are reported relative to SCE. Resonance Raman spectra were measured following published protocols (*18*). Data were collected at 90° scattering from samples in capillaries at room temperature, using 457.9 nm excitation, 20 mW power, and 10 min data accumulation. 2,4,5-Tribenzyloxybenzaldehyde was obtained from Regis. All other chemicals were obtained from Aldrich.

*3-(2,4,5-Tribenzyloxyphenyl)propenoic Acid (**5**).* Malonic acid (2.08 g) and 2,4,5-tribenzyloxybenzaldehyde (4.25 g) were dissolved in dry pyridine (20 mL). Piperidine (0.500 mL) was added, the mixture was heated at 85 °C for 2 h and then at 115 °C for 2 h. Solvent was removed *in vacuo*, and the resulting solid was suspended in a slurry of ice (100 g) and hydrochloric acid (1 M, 100 mL). The solid was collected by filtration and washed with water (200 mL) to afford 4.74 g of **5** (100%) as a white solid: IR (NaCl) 2650−3100 (br), 1684.3, 1606.7, 1517.0, 1424.1, 1021.6, 695.6 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.04 (s, 2H), 5.11 (s, 2H), 5.13 (s, 2H), 6.31 (d, 1H, J = 16.0 Hz), 6.55 (s, 1H), 7.27 (s, 1H), 7.32−7.45 (m, 15H), 8.07 (d, 1H, J = 16.0 Hz); ^{13}C NMR δ 71.1, 71.4, 72.4, 76.8, 77.0, 77.3, 101.4, 115.1, 115.6, 116.0, 127.2, 127.3, 127.5, 128.0, 128.1, 128.5, 128.6, 128.7, 136.4, 136.5, 137.0, 141.7, 143.0, 152.4, 153.5, 172.8; FABMS *m*/*z* 466; HRFABMS *m*/*z* calcd for $C_{30}H_{26}O_5$ 466.1780, found 466.1789.

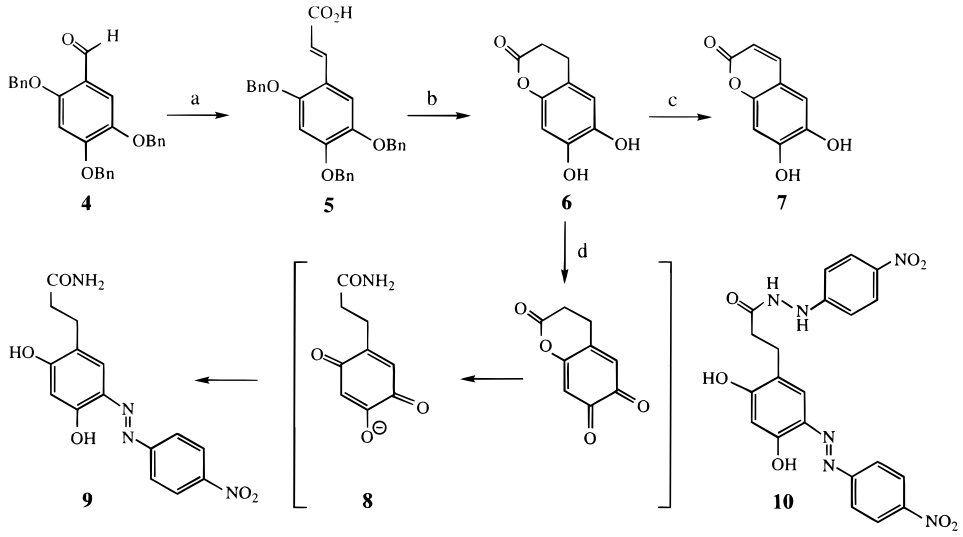
*3,4-Dihydroesculetin (**6**).* Method A: In a 250 mL Parr bottle compound **5** (0.757 g), ethyl acetate (7.54 g), and 10% palladium on carbon (75.5 mg) were hydrogenated at 50 psi for 12 h. Removal of the volatile components gave 330 mg solids which were taken up in near boiling ethyl acetate (10 mL) and filtered. Near boiling hexanes (10 mL) were added to the filtrate, and after being stored at −20 °C the solids were collected and dried to give 201 mg of **6** (69%) as transparent plates. Method B: In a 250 mL Parr bottle **5** (1.84 g), 5% palladium on carbon (96.3 mg), ethyl acetate (8.00 mL), and trifluoroacetic acid (0.250 mL) were hydrogenated at 55−60 psi for 3 h. Filtration through Celite and removal of the volatile components *in vacuo* gave a tan solid. Recrystallization from ethyl acetate gave 441 mg of **6** (62%) as white needles: IR (NaCl) 3355, 1707, 1517, 1346, 1283, 1112 cm^{-1}; UV λ_{max} (acetonitrile) 210, 272 nm; ^{1}H NMR (DMSO-d_6) δ 2.66 (dd, 2H, J = 8.0, 6.0 Hz) 2.77 (dd, 2H, J = 7.5, 6.0 Hz), 6.43 (s, 1H), 6.59 (s, 1H), 8.84 (s, 1H, exchangeable), 9.11 (s, 1H, exchangeable); ^{13}C NMR δ 22.7, 29.5, 104.4, 113.2, 114.7, 142.2, 144.4, 145.0, 169.3; HRFABMS calcd for $C_9H_8O_4$ 180.0423, found 180.0424.

*3-[2,4-Dihydroxy-5-(4-nitrophenylazo)phenyl]propanamide (**9**).* Method A: To 4-nitrophenylhydrazine (459 mg) was added sulfuric acid (2 mL, 36 N) and water (3 mL). This was heated to near boiling, then ethanol (95%, 10 mL) was added, and upon reaching ambient temperature, the mixture was filtered. In a second vessel a solution of **6** (22.7 mg) in acetonitrile (2.25 mL) was degassed and then treated with a stream of ammonia until compound **6** was no longer observed by TLC (MeOH/dichloromethane, 1:20). The volatile materials were removed, and fresh acetonitrile was added (2.25 mL). The solution was treated with oxygen gas and catalytic ethyldiisopropylamine. At 0.25 h the 4-nitrophenylhydrazine solution prepared above (1 mL) was added, and after an additional 0.5 h, the mixture was set aside for crystal formation. Filtration of the resulting crystals gave 12.1 mg of **9** (29%) as red needles. Method B: Oxygen gas was bubbled through **6** (38.2 mg) in methanol (6 mL). Ammonium hydroxide (0.100 mL) was added followed by 0.2 h by 4-nitrophenylhydrazine (41.2 mg). At 2 h this was poured into hydrochloric acid (1 M, 50 mL); then the combined material was extracted with ethyl acetate (2 × 25 mL). The extracts were washed with brine (25 mL) and dried over Na_2SO_4, and the solvent was removed to give a red gum. Recrystallization from DMF/water gave 13.3 mg of **9** (19%) as red needles: ^{1}H NMR (DMSO-d_6) δ 2.35 (t, 2H, J = 7.5 Hz), 2.71 (t, 2H, J = 7.5 Hz), 6.40 (s, 1H), 6.78 (s, 1H), 7.31 (s, 1H), 7.52 (s, 1H), 8.03 (d, 2H, J = 8.8 Hz), 8.36 (d, 2H, J = 9.0 Hz), 9.25 (s, 1H), 10.39 (s, 1H); HRFABMS *m*/*z* calcd for $C_{15}H_{15}N_4O_5$ 331.1042 $[M + H]^+$, found 331.1048.

*3-[2,4-Dihydroxy-5-(4-nitrophenylazo)phenyl]propanoic Acid (**11**).* 3-(2,4,5-Tribenzyloxyphenyl)propenoic acid (**5**) (173.8 mg, 0.373 mmol) was suspended in water (2 mL) and THF (2 mL). To this K_2CO_3 (40.2 mg, 1.08 equiv) and 5% palladium on carbon (37.7 mg) were added. After hydrogenation at 60 psi for 20 h on a Parr shaking apparatus, the reaction mixture was filtered through Celite into a flask containing a solution of 4-nitrophenylhydrazine (61.1 mg, 0.4 mmol) in THF (5 mL). This was stirred open to the air for 1 h, acidified with HCl (1 M), and extracted with ethyl acetate. Removal of the solvent *in vacuo* gave 86.8 mg of a red-orange solid which was chromatographed (1.5 × 17 cm silica) with chloroform/acetone/MeOH/HOAc (100:50:5:1) to afford after removal of solvent 20.4 mg of **11** (17%) as a red solid: ^{1}H NMR (acetone-d_6) δ 2.70 (t, 2H, J = 7.5 Hz), 2.95 (t, 2H, J = 7.0 Hz), 6.44 (s, 1H), 7.76 (s, 1H), 8.06 (dd, 2H, J = 1.5, 7.5 Hz), 8.40 (dd, 2H, J = 2.0, 7.0 Hz), 10.4 (br s, 2H), 13.22 (s, 1H); ^{13}C NMR δ 24.7, 33.1, 103.0, 121.8, 121.9, 124.9, 125.4, 134.3, 147.6, 154.2, 157.0, 162.3, 173.4; EIMS *m*/*z* 331, 313 (base peak), 191, 163; HREIMS *m*/*z* calcd for $C_{15}H_{13}N_3O_6$ 331.0804, found 331.0809.

*3,4-Dihydro-7-hydroxy-6-(4-nitrophenylazo)-1,2-benzopyrone (**12**).* The propanoic acid **11** (8.7 mg, 0.026 mmol) and

Scheme 2: Schematic for Preparation of Compounds **5**−**9** and Structure for **10**[a]



[a] Reagents and conditions: (a) $HO_2CCH_2CO_2H$, pyridine, piperidine, 80 °C; (b) H_2, Pd/C, 62%; (c) $PhI(OAc)_2$ or DDQ; (d) 1. NH_3, O_2; 2. 4-NO_2-PhN_2H_3·HCl.

1,3-dicyclohexylcarbodiimide (5.5 mg, 0.027 mmol) were suspended in dichloromethane (4 mL). To this was added 4-(dimethylamino)pyridine (3.4 mg, 0.028 mmol), and after being stirred for 17 h, the mixture was washed successively with HCl (0.5 M, 2 × 2 mL) and water (3 × 2 mL). Chromatography of the resulting material (1 × 9 cm silica) with chloroform afforded 5.1 mg solids which were recrystallized from chloroform/pentane to give 3.9 mg (4.7%) of **12** as bright red needles: UV/vis (acetonitrile) λ_{max} (ϵ) 202 (340 000), 262 (31 300), 278 (sh, 22 200), 338 (15 300), 404 nm (12 900); ^{1}H NMR ($CDCl_3$) δ 2.87 (dd, 2H, J = 5.6, 7.6 Hz), 3.09 (t, 2H, J = 6.4 Hz), 6.74 (s, 1H), 7.83 (s, 1H), 7.98 (dd, 2H, J = 2.4, 7.0 Hz), 8.39 (dd, 2H, J = 2.2, 7.2 Hz), 12.90 (s, 1H); ^{13}C NMR δ 23.0, 29.2, 106.4, 115.5, 122.7, 125.0, 133.3, 135.0, 148.6, 153.9, 154.2, 156.4, 167.0; EIMS *m*/*z* 313 (base peak), 283, 224, 191, 163; HREIMS *m*/*z* calcd for $C_{15}H_{11}N_3O_5$ 313.0699, found 313.0704.

*4-(4-Nitrophenylazo)-6-ethylresorcinol (**14**).* To a solution of 4-ethylresorcinol (2.76 g, 20 mmol) in aqueous NaOH (10 M, 5 mL) was added a solution of $NaNO_2$ (1.52 g, 22 mmol) in water (5 mL). In a 50 mL Erlenmeyer flask 4-nitroaniline (3.32 g, 24 mmol) was suspended in aqueous HCl (15 mL) and water (5 mL) by sonication. The solution containing the 4-ethylresorcinol was added dropwise over 0.1 h. After being stirred for 1 h, the precipitate was collected by filtration, washed sequentially with HCl (1 M) and water, and then dried *in vacuo* to give 4.13 g of **14** (72%) as a dark red solid. An analytical sample was prepared by recrystallization from DMF/water: ^{1}H NMR (DMSO-d_6) δ 1.13 (t, 3H, J = 7.5 Hz), 2.45−2.49 (m, 2H, coincidental with DMSO-d_6), 6.35 (s, 1H), 7.43 (s, 1H), 7.95 (d, 2H, J = 8.5 Hz), 8.29 (d, 2H, J = 8.8 Hz), 9.00 (s, 1H, exchangeable), 10.46 (s, 1H, exchangeable); ^{13}C NMR δ 13.7, 21.9, 102.7, 121.7, 125.0, 125.6, 126.7, 133.2, 146.5, 154.1, 158.8, 163.7; EIMS *m*/*z* 287.165 (base peak), 137; HREIMS *m*/*z* calcd for $C_{14}H_{13}N_3O_4$ 287.0906, found 287.0908.

*4-(4-Nitrophenylazo)-6-ethyl-1-butyrylresorcinol (**15**).* To a solution of 4-(4-nitrophenylazo)-6-ethylresorcinol (**14**) (102 mg, 0.36 mmol) in THF (5 mL) was added butyric anhydride (63.9 μL, 1.10 equiv). This was followed by triethylamine (49.5 μL, 1 equiv). The mixture was stirred at ambient temperature overnight and then partitioned between water (10 mL) and chloroform (10 mL). The organic layer was washed sequentially with aqueous HCl (1 M, 5 mL), water (2 × 5 mL), and brine (5 mL). After removal of the chloroform *in vacuo*, the resulting solid was chromatographed (1.5 × 18 cm silica gel) with chloroform/hexanes (2:1) to give after removal of solvent 18.6 mg. This material was recrystallized from chloroform/hexanes to give 13.5 mg of **15** (11%) as bright red needles: UV/vis (acetonitrile) λ_{max} (ϵ) 200 (sh, 146 000), 262 (36 400), 278 (sh, 27 000) 332 (15 700), 404 nm (8600); ^{1}H NMR ($CDCl_3$) δ 1.08, 1.27 (t, 3H, J = 7.4 Hz), 1.80−1.86 (m, 2H), 2.57−2.62 (m, 4H), 6.78 (s, 1H), 7.68 (s, 1H), 7.98 (dd, 2H, J = 7.1, 2.0 Hz), 8.38 (dd, 2H, J = 6.9, 2.0 Hz), 12.69 (s, 1H); ^{13}C NMR δ 13.7, 13.9, 18.4, 22.3, 36.2, 112.0, 122.7, 124.9, 125.0, 28.8, 134.2, 136.2, 148.5, 152.4, 154.0, 171.3; EIMS *m*/*z* 357, 287 (base peak), 165, 137; HREIMS *m*/*z* calcd for $C_{18}H_{19}N_3O_5$ 357.1325, found 357.1329.

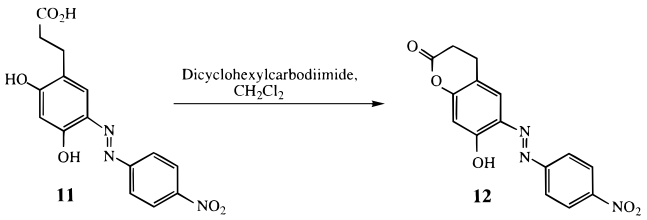
4-Nitrophenylhydrazones of TPQ Model Compounds. These were prepared according to published procedures (*4*).

RESULTS AND DISCUSSION

The synthesis of the acyl-substituted model compounds began with the tribenzyloxyaldehyde (**4**, Scheme 2), which was condensed with malonic acid to afford **5**. This material was hydrogenated, deprotected, and annulated in one synthetic step to afford 3,4-dihydroesculetin (**6**) (*19*). This compound has the structure of the proposed native cofactor but with the side chains abbreviated in the δ-lactone. Attempted oxidation of this compound to the corresponding *o*-quinone with iodobenzene diacetate or 2,3-dichloro-5,6-dicyano-1,4-benzoquinone gave the dehydrogenation product esculetin (**7**). Compound **7** may be generated by way of a quinone methide followed by tautomerization. Autoxidation of **6** in the presence of ammonia gave a ring-opened product derived from the putative *o*-quinone **8** that was trapped as its 4-nitrophenylhydrazine derivative **9**.

These experiments suggested that the quinone oxidation level of **6** may be less accessible than that of TPQ or LTQ.

Scheme 3: Schematic for Preparation of Compound **12**

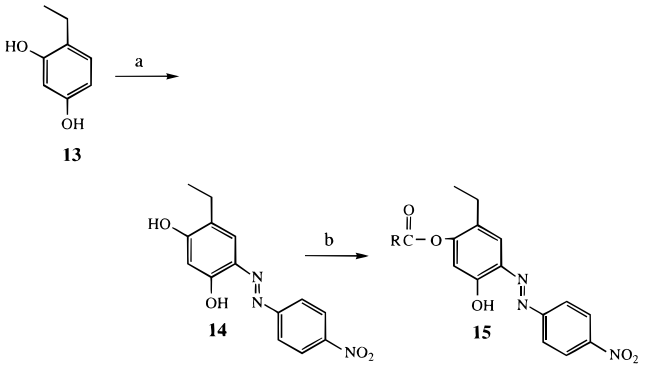


To address this possibility, the redox potential of **6** was determined by cyclic voltammetry. Fresh solutions of **6** were colorless and gave a single signal centered at $E_m = +133$ mV vs SCE. This indicates that the oxidation of **6** requires a much more positive potential that do the model compounds for TPQ [$E_m = -150$ mV at pH 6.8(4)] and LTQ [$E_m = -182$ mV at pH 7.0 (20)]. Solutions of **6** in pH 6.70 phosphate buffer became pink over a short time. Cyclic voltammetry of these pink solutions showed a new $E_m = -149$ mV in addition to that for **6**. The intensity of this new signal increases with time and corresponds to the solvolysis of the lactone to give the corresponding carboxylate, bearing a TPQ substructure. We note that quino-cofactor electrochemistry has been focused thus far on the oxygen-substituted cofactor models rather than the biologically relevant anilines. For the future, the corresponding anilines should be synthetically accessible, for instance, by hydrogenolysis of the azo compounds.

Characterization of the enzyme-bound TPQ, LTQ, and the AO-I cofactors has been facilitated by derivatization of protein with phenylhydrazines, often 4-nitrophenylhydrazine (*21*). These derivatives enhance stability and provide a characteristic UV/vis chromophore that can be used for resonance Raman studies. For this reason, a 4-nitrophenylhydrazine derivative of structure **3** (Figure 1) was pursued. Autoxidation of **6**, catalyzed by ammonia, and trapping of the intermediate quinone with 4-nitrophenylhydrazine gave the desired substitution at C-5, accompanied by an undesired attack of ammonia at the carbonyl carbon to give the azo amide **9**. Similarly, using 4-nitrophenylhydrazine to catalyze the oxidation directly gave the azo hydrazide **10**. To ascertain if the δ-lactone could be re-formed, amide **9** was treated with a variety of protic and Lewis acids. However, amide **9** was unreactive in all conditions surveyed. This suggested that it would be necessary to generate a derivatized TPQ structure prior to acylation via ring closure.

With this is mind we turned our attention to **5**, recognizing that the carboxylic acid could be protected from amidation by forming its salt. Thus, the potassium salt of **5** was hydrogenated and deprotected as before but then directly oxidized with O_2 in the presence of 4-nitrophenylhydrazine to give the azo carboxylic acid **11** (Scheme 3) directly upon acidification. When treated with dicyclohexylcarbodiimide, compound **11** ring closed to give the desired δ-lactone **12**. We noticed that the longest UV/vis λ_{max} of this compound (404 nm) was in poor agreement with that reported for the 4-nitrophenylhydrazine-labeled enzyme AO-I (465 nm). While the magnitude of this difference suggested the active site of AO-I did not contain the originally proposed structure, we wanted to be certain that the difference in absorption was not due to the acyl substituent being rotationally constrained.

Scheme 4: Schematic for Preparation of Compounds **14** and **15**[a]



[a] Reagents and conditions: (a) $NaNO_2$, 4-NO_2-$PhNH_2$·HCl; (b) butyric anhydride, THF.

To test this hypothesis, resorcinol **13** was coupled with 4-nitrophenyldiazonium chloride to provide the azo-TPQ derivative **14** (Scheme 4). In this and other azo resorcinol derivatives, the phenolic hydroxyl group adjacent to the azo substituent is expected to be strongly hydrogen bonded to nitrogen (10.46 ppm). This provides a ready means of assigning the phenolic protons in the NMR spectrum and, thus, the extent of selective reactivity at the 1- vs 3-hydroxyl groups in **14**. Treatment of compound **14** with butyric anhydride gave compound **15**. The regiochemistry of acylation was evident from the retention of a hydrogen-bonded phenol in the ^1H NMR and the ^{13}C chemical shift of the product. Once again, the longest UV/vis λ_{max} was 404 nm, in contrast to that reported for the proposed cofactor.

As a final structural probe of our models for the active site cofactor in AO-I, we turned to resonance Raman spectroscopy. Resonance Raman has proven to be a powerful tool for structural assignment of quino-cofactors derivatized with phenylhydrazines (*24*). In the original study of the AO-I active site structure, derivatization with 4-nitrophenylhydrazine was reported to lead to a resonance Raman spectrum (*16*) that was virtually identical to spectra reported earlier for the same derivative of TPQ in other enzymes (*25*). Shown in Figure 2 are resonance Raman spectra for 4-nitrophenylhydrazine derivatives of TPQ model compounds that differ solely with regard to their alkyl side chain. In all cases, the spectra in Figure 2 indicate similar frequencies and relative intensities: in particular, there is the characteristic band near 1598−1599 cm^{-1}, the pair of peaks near 1341 and 1388 cm^{-1}, and the pair of peaks at 1158 and 1110 cm^{-1}. The Raman spectral intensity pattern is similar to that reported for the enzyme derivatives (*25*), but the frequencies are ca. 7 cm^{-1} higher due to the change from an aqueous environment for the enzymes to $CDCl_3$ for the model compounds. As shown in Figure 3, a totally different pattern of peak frequencies and intensities appears for the 4-nitrophenylhydrazine derivatives of the model compounds prepared to mimic the active site structure proposed for AO-I: the dominant spectral feature arising from the TPQ carboxylate esters is a set of four peaks centered at 1426, 1344, 1273, and, most prominently, 1397 cm^{-1}. These data establish that, as expected, resonance Raman spectroscopy is sensitive to the nature of the substituent [OH vs O−C(=O)−R] at the 5-position of the quinone ring.

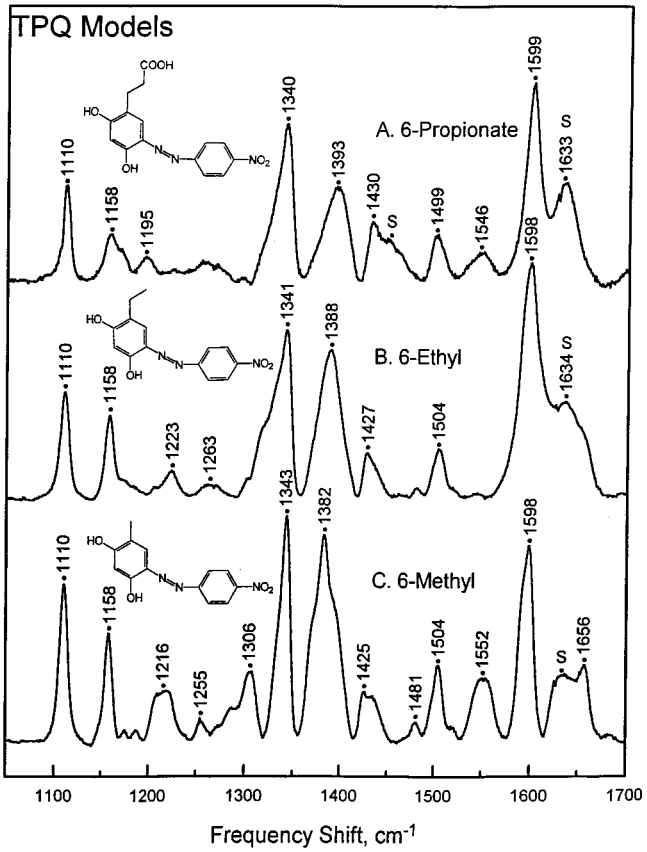


FIGURE 2: Resonance Raman spectra for 4-nitrophenylhydrazine derivatives of TPQ model compounds. S indicates spectral contribution from solvent.

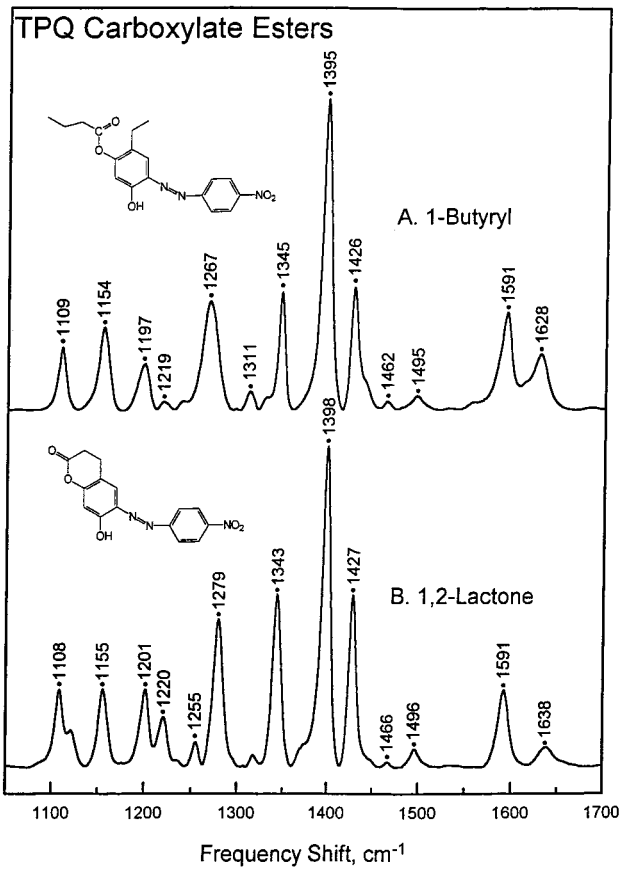


FIGURE 3: Resonance Raman spectra for 4-nitrophenylhydrazine derivatives of TPQ carboxylate ester model compounds.

CONCLUSIONS

There are numerous properties of the model compounds for the TPQ carboxylate esters and their derivatives that argue against the presence of structure **3** (Figure 1) at the active site of AO-I. From a spectroscopic perspective, there is the blue shift in the λ_{max} of the 4-nitrophenylhydrazone of the model compounds (**12** and **15**) in relation to the 4-nitrophenylhydrazone of AO-I (*16*). More compellingly, the resonance Raman spectrum for the 4-nitrophenylhydrazone of AO-I (*16*) is strikingly similar to that for TPQ (Figure 2) and different from that for the 4-nitrophenylhydrazone of two TPQ carboxylate esters (Figure 3). The redox potential for compound **6** also argues against a catalytic role for this structure in an enzyme-catalyzed reaction. In the case of biologically active quino-cofactors whose structures have been well established, redox potentials of −155 mV at pH 6.68 [pyrroloquinoline quinone (*22*)], −150 mV [TPQ (*4*)], −182 mV [LTQ (*20*)], and −150 mV at pH 6.8 [tryptophan tryptophanyl quinone (*23*)] have been reported. In contrast, the redox potential for compound **6** is considerably more positive (+133 mV). While this alone does not rule out a role for a TPQ carboxylate ester as an enzymatic cofactor, it is in striking contrast to the redox properties of all previously characterized, functional quino-cofactors. Finally, there is the issue of the stability of the carboxylate ester. In the course of cyclic voltammetric studies, the quinone with an ester linkage at position 2 underwent hydrolysis to generate a TPQ-like model compound at near physiological pH (pH 7.6). This does not appear to be due to strain in the lactone ring, since spectral characterization of an acyclic ester derivative indicates the same λ_{max} for its 4-nitrophenylhydrazone derivative as seen for compound **12**.

In conclusion, the studies presented herein argue that the active site cofactor in AO-I is TPQ and not a TPQ carboxylate ester. The data also indicate that a cofactor in which a carboxylate side chain is cross-linked to the quinone ring lacks the properties anticipated for catalytic function. For the future, it will be very interesting to prepare and characterize alternate variants of TPQ that contain electron-releasing groups at the 2-position of the quinone ring. Derivatives of this nature may possess the redox and stability properties that would support their participation in biological catalysis.

ACKNOWLEDGMENT

We thank Dr. Benjamin Schwartz and Dr. Minae Mure for careful reading of the manuscript and helpful comments.

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BI000507M