Model Studies Support Pyrrolylation of the Topaquinone Cofactor To Explain Inactivation of Bovine Plasma Amine Oxidase by 3-Pyrrolines. Unusual Processing of a Secondary Amine

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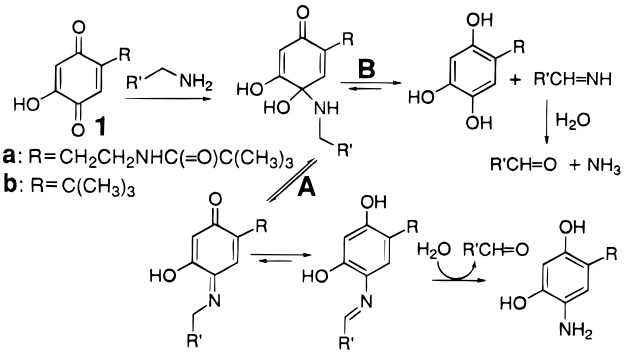
Received December 29, 1995

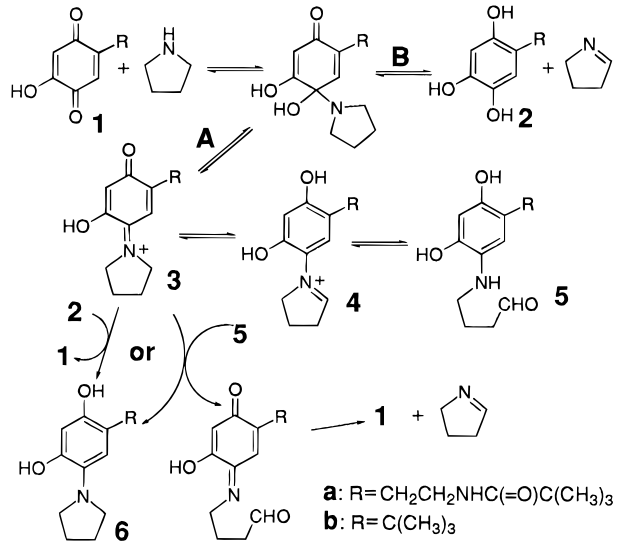
Since the report in 1990 that the active carbonyl cofactor used by plasma and other copper amine oxidases is the quinone form (topaquinone, TPQ) of an active site 2,4,5-trihydroxyphenylalanine residue,[1] there has been renewed interest in mechanistic and directed model studies.[2−7] A pyridoxal-like transamination mechanism appears to be in force for the enzyme (Scheme 1, path A), since anaerobic single turnover results in release of aldehyde product, whereas NH_3 is released only upon O_2-dependent reoxidation of the reduced cofactor.[8] In contrast, an addition−elimination mechanism (Scheme 1, path B), found to be a competing pathway for benzylamine deamination using pyrroloquinoline quinone as a model,[9] would predict simultaneous release of aldehyde and NH_3 after anaerobic single turnover. The fact that secondary amines are not substrates for the enzyme[10] might be rationalized on the basis that transamination in this case would require tautomerization between two iminium intermediates (see Scheme 2). However, we here communicate model study results demonstrating the occurrence of transamination for a secondary amine that led us to detect enzymatic processing in the form of a mechanism-based inactivation event.

We recently reported a pivalamidoethyl-based model **1a** which is active in the catalytic aerobic deamination of benzylamine in buffered *aqueous* acetonitrile,[4] whereas Mure and Klinman investigated the ability of various topaquinone models to catalyze aerobic deamination in *anhydrous* acetonitrile.[5] These initial model studies revealed several features of relevance to the enzymes, but the observation of catalytic turnover did not permit a distinction between transamination and addition−elimination mechanisms. In fact, although the spectrophotometric behavior of a TPQ model reaction with benzylamine under pseudo-first-order conditions was shown to be consistent with a transamination mechanism,[6] results for $PhCD_2NH_2$ compared to those for $PhCH_2NH_2$ revealed that the spectrophotometric data could not provide an unambiguous mechanistic interpretation due to overlapping of "substrate" (quinone) imine and "product" (aldehyde) imine chromophores.[7]

Scheme 1



Scheme 2



We hoped that a mechanistic distinction could be made simply by determining whether the product of anaerobic single turnover was the aminoresorcinol (from transamination) or benzenetriol (from addition−elimination) (see Scheme 1). However, the occurrence of "redox interchange" reactions, which scrambled the nature of the initial product of quinone reduction as soon as it was formed, prevented a mechanistic conclusion based on product analysis, even upon continuous NMR monitoring of the reaction.[4] A solution to this dilemma was found (i) by manipulating NMR reaction conditions to favor the unimolecular throughput of the initial quinone−amine adduct at the expense of bimolecular redox interchange events and (ii) by analyzing products of a diagnostic substrate that was devoid of redox interchange complications.[7] In this way we were able to demonstrate unambiguously a preferred transamination mechanism.[7]

At this point, we believed that a secondary amine such as the sterically "tied back" pyrrolidine might recruit an addition−elimination pathway (Scheme 2, path B) in our model system. However, our finding that reactions of pyrrolidine with both **1a** and the *tert*-butyl-based model **1b**[5] in CD_3CN led to the

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(5) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8698.
(6) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8707.
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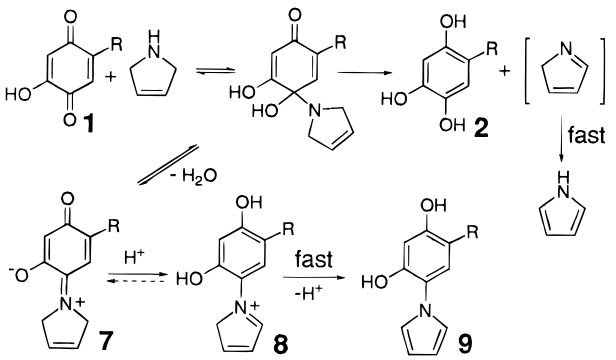
(8) Janes, S. M.; Klinman, J. P. *Biochemistry* **1991**, *30*, 4599.
(9) (a) Ohshiro, Y.; Itoh, S.; *Bioorg. Chem.* **1991**, *19*, 169. (b) Rodriguez, E. J.; Bruice, T. C. *J. Am. Chem. Soc.* **1989**, *111*, 7947.
(10) A recent report (Houen, G.; Bock, K.; Jensen, A. L. *Acta Chem. Scand.* **1994**, *48*, 52) suggests that the polyamines spermine and spermidine are preferentially metabolized by copper amine oxidases at the secondary amine positions. This conclusion was based on (i) the authors' inability to identify the (amino)dialdehydes expected from primary amine deamination and (ii) the identification instead of 3-aminopropanal, the product expected from secondary amine metabolism. However, our reading of the Houen et al. paper suggests their inability to detect the (amino)dialdehydes reflects their failure to recognize the earlier reported[11] need to achieve rapid enzymatic reaction so that the initial (amino)dialdehydes can be reductively stabilized (BH_4^-) prior to their decomposition. This decomposition is a retro Michael reaction producing acrolein, which we have found can combine, under the reaction conditions, with the NH_3 generated from terminal deamination, thereby rationalizing the 3-aminopropanal detected. Thus, as will be described elsewhere, we support the earlier interpretation[11] that polyamines are metabolized at primary and not secondary amine positions.
(11) Tabor, C. W.; Tabor, H.; Bachrach, U. *J. Biol. Chem.* **1964**, *239*, 2194.

S0002-7863(95)04321-6 CCC: $12.00

Scheme 3



generation of the redox interchange product, (pyrrolidino)-resorcinol **6**, as the major product,[12] could only be understood in terms of generation of the corresponding quinone*iminium* intermediate **3**, which was being reduced by benzenetriol **2** or by (alkylamino)resorcinol **5**. The existence of **3** implied that transamination (Scheme 2, path A) might still be the predominant reaction pathway, and we were in fact able to detect (^{1}H NMR, D_2O) the subsequent iminium intermediate **4** following aqueous HCl quenching of the reaction of pyrrolidine with **1a**.[13]

We then recognized that upon replacing pyrrolidine with the activated (doubly allylic) analog 3-pyrroline, transamination and addition−elimination pathways would be distinguished by whether the observed product was pyrrole or the pyrrolated cofactor **9** (Scheme 3). Redox interchange complications in this case would presumably be thwarted on account of rapid irreversible tautomeric aromatizations. In fact, reaction of **1b** with 3-pyrroline in CD_3CN was found to give exclusively **9b**.[14]

This result suggested to us that 3-pyrroline might act as a cofactor-directed irreversible inactivator of TPQ-dependent amine oxidases with little if any productive turnover. In fact, no O_2 uptake could be detected when bovine plasma amine oxidase (BPAO, from Sigma) was incubated with up to 10 mM pyrrolidine or 3-pyrroline,[15] consistent with the alleged non-substrate behavior of secondary amines. However, preincubation with 3-pyrroline resulted in a concentration- and time-dependent pseudo-first-order loss of BPAO activity.[15] Kitz and Wilson analysis of the first-order inactivation rate constants at 30 °C, pH 7.2 yielded values of $K_i = 50$ mM and $k_{inact} = 0.3$ min^{-1}. The rather high K_i for 3-pyrroline is consistent with very weak binding for this simple aliphatic amine (the preferred substrates for BPAO are *aryl*alkylamines), *but the clear processing that occurs nonetheless constitutes what appears to be the first precedent*[10] *for enzymatic processing of a secondary amine*. We suggest that the usual nonsubstrate behavior of typical secondary amines toward the copper amine oxidases is active site steric exclusion rather than their inability to undergo transamination. In this regard, it is clear that mechanism-based inactivation serves as a much more sensitive test of enzymatic processing than does substrate activity.

In an effort to improve the inhibitory efficiency of 3-pyrroline, we considered adding an aryl ring "recognition element" to give 3-phenyl-3-pyrroline. This compound[16] displayed a more potent concentration- and time-dependent pseudo-first-order loss of BPAO activity (50% inhibition was observed in 30 min at 0.1 mM) that was reduced in the presence of substrate benzylamine. A Kitz and Wilson plot of the inactivation rate data in this case comes too close to the O−O origin to permit an accurate estimate of the intercepts. We suspect that the apparent lack of saturation (see ref 17) merely reflects a more efficient turnover (inactivation) at the expense of an insufficiently improved binding. The inactivation is thus instead described as a second-order rate constant, 230 M^{-1} min^{-1}, which can be compared to $k_{inact}/K_i = 6$ M^{-1} min^{-1} for 3-pyrroline itself.

Inhibition by the 3-pyrrolines was found to be irreversible as indicated by reassay of activity following Sephadex G-25 chromatography and was accompanied by loss of the 480 nm chromophore ascribed to the TPQ cofactor. The latter finding, along with our inability to see the normal development at A_{450} following titration with phenylhydrazine, is consistent with our proposed cofactor pyrrolylation mechanism (Scheme 2). It will be of obvious interest to confirm for the enzyme that the cofactor has actually been pyrrolylated in the manner we see in the model reaction.

Further analog development may lead to even more efficient inactivators in the 3-pyrroline class, and it will be important to determine if our results here generalize to other enzymes in the TPQ-dependent family of copper amine oxidases. Presuming that inactivation reflects cofactor pyrrolylation, 3-pyrrolines represent examples of *transamination-specific* inactivators; that is, for enzymes such as the flavin-dependent mitochondrial amine oxidase which utilize nontransamination mechanisms for amine oxidation, the 3-pyrrolines should simply be turned over as normal substrates to pyrroles. We hope to exploit this potential mechanistic distinction in the development of selective inhibitors of the TPQ-containing enzymes.

Acknowledgment. We are grateful to NIH for support of this work through Grant GM-48812.

Supporting Information Available: Text giving experimental details for the syntheses, chemical model reactions, and enzymologic studies and kinetic plots of inactivation (6 pages). See any current masthead page for ordering and Internet access instructions.

JA9543210

(12) The reaction of 50−60 mM **1a** or **1b** and 150−180 mM pyrrolidine in degassed CD_3CN was monitored by ^{1}H NMR spectroscopy. After 2 days (no further reaction), the major product was **6a** or **6b**, identified by their independent "redox cycling" synthesis[3] from triol **2a** or **2b** and pyrrolidine. **6a**: ^{1}H NMR (CD_3CN, 300 MHz) δ 1.11 (s, 9H), 1.89 (m, 4H), 2.62 (t, 2H, $J = 7.08$ Hz), 2.92 (m, 4H), 3.23 (m, 2H), 6.35 (s, 1H), 6.67 (br s, NH, 1H), 6.82 (s, 1H). ^{13}C NMR (CD_3CN, 75 MHz) δ 25.0 (2C), 27.7 (3C), 30.7, 39.1, 41.0, 53.7 (2C), 102.7, 117.3, 123.2, 130.5, 152.1, 153.2, 180.0. **6b**: ^{1}H NMR (CD_3CN, 300 MHz) δ 1.31 (s, 9H), 1.90 (m, 4H), 2.92 (m, 4H), 6.28 (s, 1H), 7.00 (s, 1H). ^{13}C NMR (CD_3CN, 75 MHz) δ 25.03 (2C), 30.08 (3C), 34.90, 53.91 (2C), 103.28, 120.03, 128.09, 129.67, 151.50, 153.30. HRMS (EI) calcd for $C_{14}H_{21}NO_2$ *m/z* 235.1573; found 235.1579 (56.7%). The major side product in the case of **1b** was identified, following isolation, as that arising from electrophilic substitution of 1-pyrroline at C-2 of **6b**, 2-(pyrrolidin-2-yl)-4-(pyrrolidin-1-yl)-6-*tert*-butylresorcinol.

(13) Signals corresponding to **4a** seen during ^{1}H NMR spectral monitoring of the reaction of pyrrolidine with **1a** were identified through independent generation of **4a** by aqueous HCl treatment of the diethylacetal of the precursor 4-anilinobutanal, in turn prepared by redox cycling[3] reaction of **2a** with 4,4-diethoxybutanamine.

(14) The reaction of 160 mM **1b** and 250 mM 3-pyrroline in degassed CD_3CN was monitored by ^{1}H NMR spectroscopy. Compound **9** was formed quantitatively in 2 h: ^{1}H NMR (CD_3CN, 300 MHz) δ 1.34 (s, 9H), 4.83 (bs, OH, 2H), 6.18 (t, 2H, $J = 2.14$ Hz), 6.43 (s, 1H), 6.85 (t, 2H, $J = 2.14$ Hz), 6.98 (s, 1H). ^{13}C NMR (CD_3CN, 75 MHz) δ 29.88 (3C), 34.61, 105.61, 109.13 (2C), 122.95 (2C), 125.49, 128.43, 128.84, 150.25, 155.87. HRMS (EI) calcd for $C_{14}H_{17}NO_2$ *m/z* 231.1260; found 231.1260 (88.7%).

(15) Bovine plasma amine oxidase (Sigma, 40−80 units/gram of protein) was incubated with various concentrations of inhibitors (pH 7.2, 30 °C), and aliquots were assayed for activity by determining the rate of oxidation of benzylamine (10 mM) to benzaldehyde at 250 nm. O_2 uptake was monitored using a Yellow Springs Instruments 5300 biological oxygen meter at 25 °C, pH 7.2 using 0.7 μM BPAO in a total volume of 2 mL. Under these conditions, complete consumption of O_2 occurred in 20 min using 10 mM benzylamine.

(16) 3-Phenyl-3-pyrroline was prepared by addition of PhMgBr to *N*-(carboethoxy)-3-pyrrolidone, N-deprotection of the resulting phenyl carbinol by refluxing overnight in 1:1 *n*-propanol aqueous KOH (10 N), and finally dehydration by refluxing in concd HCl for 1 h. The compound has also been prepared from 2-phenyl-2-vinylaziridine: Hortmann, A. G.; Koo, J.-y. *J. Org. Chem.* **1974**, *39*, 3781.

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