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Articles

Temporary Inactivation of Plasma Amine Oxidase by Alkylhydrazines. A Combined Enzyme/Model Study Implicates Cofactor Reduction/Reoxidation but Cofactor Deoxygenation and Subsequent Reoxygenation in the Case of Hydrazine Itself

Younghee Lee, Heung-Bae Jeon, He Huang, and Lawrence M. Sayre*,†

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

LMS3@po.cwru.edu

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It has been known for some time that hydrazine and its methyl and 1,1-dimethyl analogues induce inactivation of the copper-containing quinone-dependent plasma amine oxidase but that the activity recovers over time, suggesting metabolism of all three inhibitors. However, the mechanism responsible for loss and regain of activity has not been investigated. In this study a combination of enzyme studies under a controlled atmosphere along with model studies using 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone to mimic the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor of the enzyme suggest that regain of enzyme activity represents two different O_2-dependent processes. In the case of methylhydrazine and 1,1-dimethylhydrazine, we propose that the inactive methylhydrazone/azo form of the enzyme slowly rehydrates and eliminates MeN=NH to give the triol cofactor form, which instantly reoxidizes to the catalytically active quinone form in the presence of O_2. Metabolism of methylhydrazine represents its conversion to CH_4 and N_2, and of 1,1-dimethylhydrazine to CH_2=O, CH_4, and N_2. In the case of hydrazine itself, however, we propose that the inactive hydrazone/azo form of the enzyme instead undergoes a slow decomposition, probably facilitated by the active-site copper, to give N_2 and a novel 5-desoxy resorcinol form of the cofactor. The latter undergoes a rapid, but noninstantaneous reoxygenation at C5 to restore the active cofactor form, also probably mediated by the active-site copper.

Introduction

The copper-containing quinone-dependent amine oxidases represent an important class of prokaryotic and eukaryotic enzymes responsible for the conversion of primary amines to the corresponding aldehydes.[1] The oxidative deamination half-reaction is mediated by the quinone form (TPQ) of a 2,4,5-trihydroxyphenylalanine (TOPA) residue at the active site,[2] through initial formation of the "substrate Schiff base" at the electrophilic C5 carbonyl of the TPQ cofactor, establishment of an aromatic (alkylamino)resorcinol through imine shift to the "product Schiff base", and then hydrolysis to release aldehyde and the reductively aminated cofactor (Scheme 1). Since the imine shift step is analogous to one-half the

† Phone: (216) 368-3704. Fax: (216) 368-3006.

(1) (a) Mondovi, B.; Riccio, P. *Adv. Inorg. Biochem.* **1984**, *6*, 225−244. (b) Klinman, J. P. *Chem. Rev.* **1996**, *96*, 2541−2561.

(2) Brown, D. E.; Mcguirl, M. A.; Dooley, D. M.; Janes, S. M.; Mu, D.; Klinman, J. P. *J. Biol. Chem.* **1991**, *266*, 4049−4051.

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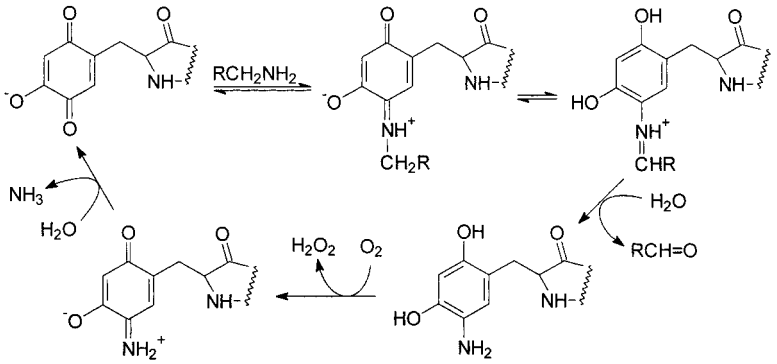
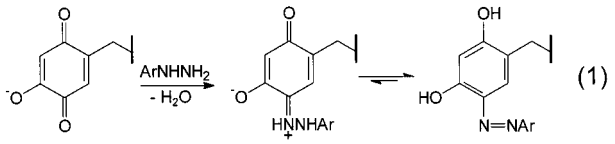
Scheme 1



transamination cycle (from pyridoxal to pyridoxamine) catalyzed by pyridoxal phosphate-dependent transaminases, the primary amine → aldehyde step mediated by the TPQ-dependent amine oxidases is also referred to as an aminotransferase or transamination process. In the second half-reaction, the reduced cofactor is reoxidized to the starting quinone concomitant with reduction of O_2 to H_2O_2 and hydrolytic release of ammonia (Scheme 1).

One of the key characteristics of the quinone cofactor of copper amine oxidases is its high reactivity toward so-called "carbonyl reagents", in particular hydrazines and hydrazides. In fact, identification of the organic cofactor of bovine plasma amine oxidase (BPAO) relied in part on isolation of a radiolabeled peptide upon digestion of the protein following derivatization with [^{14}C]phenylhydrazine.[3] Also, the ability to achieve stoichiometric titration of TPQ-dependent enzymes with phenylhydrazine, monitored by the strong absorption at near 450 nm, has become common practice for assessing the viability of the TPQ cofactor. The TPQ−hydrazine adducts can exist either as hydrazone or azo tautomeric forms, though studies using synthetic cofactor models suggested that the arylhydrazine adducts exist predominantly in their azo forms (eq 1),[4,5] as a consequence of the conjugation



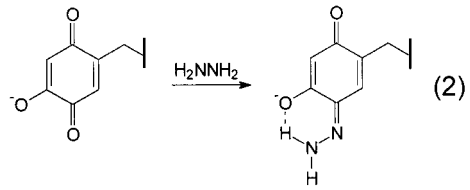
(1)

achieved. Moreover, semiempirical molecular orbital calculations for the TPQ phenylhydrazine azo derivative predict an absorption at 440 nm.[6] Thus, the irreversibility of arylhydrazine derivatization can be ascribed to the stability of the conjugated azo structure.

In contrast to *aryl*hydrazines, it was reported three decades ago that whereas reaction of BPAO with an excess of hydrazine itself and certain *alkyl*hydrazines resulted in formation of inactive complexes, these complexes readily decomposed to active enzyme and products incapable of inhibiting the enzyme.[7] The complexes could be obtained free of excess hydrazine by rapid ultrafiltration, and covalent attachment in the case of 1,1-dimethylhydrazine was demonstrated by isolation of a ^{14}C-labeled protein by gel filtration using the ^{14}C-labeled compound. Recovery of activity for the isolated complexes followed first-order kinetics, where the half-life varied with the structure of the hydrazine.[7] That full recovery of enzyme activity could be achieved through several cycles of hydrazine treatment[7] implicates metabolism of the hydrazine compound rather than simply reversal of the derivatization.

In more recent studies, it was shown that benzylhydrazine acts as a pseudo-substrate of BPAO, leading eventually to O_2-dependent benzaldehyde release and formation of what appeared spectroscopically to be the same BPAO−hydrazine adduct generated using hydrazine itself.[6,8] The authors initially proposed a mechanism in terms of pyrroloquinoline quinone (PQQ), the presumed cofactor at the time, involving C_α autoxidation of the benzylic azo derivative and breakdown of the resulting hydroperoxide to give PhCH=O, H_2O_2, and the hydrazine derivative.[8] This mechanism was later revised in terms of the TPQ cofactor.[6]

In contrast to the arylhydrazine−TPQ adducts described to exist as the azo tautomers, the hydrazine adduct of BPAO (generated directly or by decomposition of the benzylhydrazine adduct) exhibited absorptions at 335 (strong) and 400 (weak) nm and was described (supported by calculations) in terms of the hydrogen-bonded hydrazone rather than the azo tautomer (eq 2).[6]



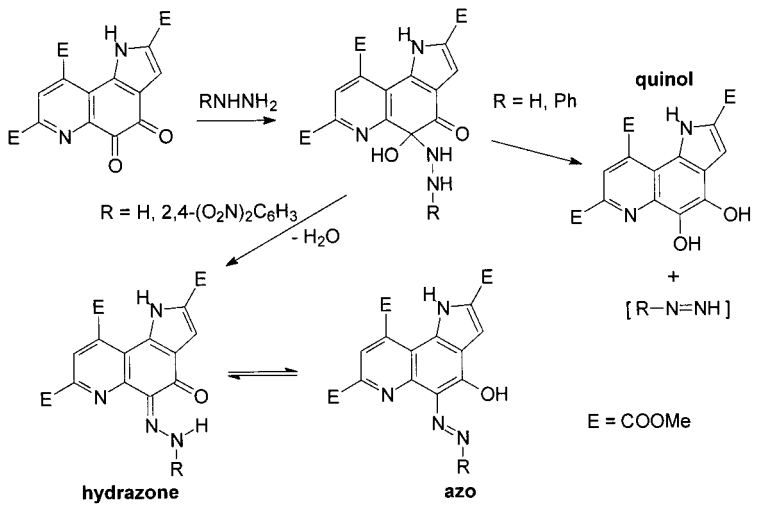
(2)

Supporting evidence is that BPAO inactivated by 1,1-dimethylhydrazine exhibited a very similar spectrum, and this derivative *can only exist as the hydrazone*.[9]

In our own studies on the synthesis of candidate primary amine substrates and inhibitors of BPAO via a Gabriel-like synthesis, which used hydrazine for deprotection of the penultimate phthalimides, we found that residual hydrazine present in the final recrystallized HCl salts, at levels undetected by combustion analysis, mani-

(3) Janes, S. M.; Mu, S.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990**, *248*, 981−987.
(4) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 7117−7127.
(5) Wang, F.; Bae, J. Y.; Jacobson, A. R.; Lee, Y.; Sayre, L. M. *J. Org. Chem.* **1994**, *59*, 2409−2417.
(6) Bossa, M.; Morpurgo, G. O.; Morpurgo, L. *Biochemistry* **1994**, *33*, 4425−4431.
(7) Hucko-Haas, J. E.; Reed, D. J. *Biochem. Biophys. Res. Commun.* **1970**, *39*, 396−400.
(8) Morpurgo, L.; Agostinelli, E.; Muccigrosso, J.; Martini, F.; Mondovi, B.; Avigliano, L. *Biochem. J.* **1989**, *260*, 19−25.
(9) De Biase, D.; Agostinelli, E.; De Matteis, G.; Mondovi, B.; Morpurgo, L. *Eur. J. Biochem.* **1996**, *237*, 93−99.

Scheme 2



fested a transient enzyme inactivation.[10] Since the mechanism responsible for transient inactivation by hydrazine and its simple methylated derivatives had never been delineated, we decided to reinvestigate their reaction with BPAO together with the use of model studies to provide clues as to the mechanism of inactivation/reactivation.

Although only the reactions of *aryl*- and not *alkyl*hydrazines with TPQ models have previously been reported,[4,5] it is instructive to examine the results of model studies conducted for both hydrazine types using (esterified) pyrroloquinoline quinone (PQQ), thought at one time to represent the BPAO cofactor.[11−14] These reactions (Scheme 2) have been shown to lead to either hydrazone/azo derivatization (2,4-dinitrophenylhydrazine),[11] reduction to the corresponding quinol (methylhydrazine and phenylhydrazine),[11,12] or a combination of both pathways (4-nitrophenylhydrazine).[11,13] Reduction of PQQ to the quinol by hydrazine itself has also been reported.[14] In general, reduction arises from elimination of RN═NH from the hydrazinocarbinol intermediate,[11,14] whereas the hydrazone results from loss of H_2O and is not an intermediate in the reduction process.[14] The PQQ model results suggested that for TPQ models, in contrast to the azo derivatives observed for *aryl*hydrazines,[4,5] for hydrazine itself, and *alkyl*hydrazines, possible quinone reduction pathways needed to be considered.

We report here that hydrazine is an unusually potent metabolism-dependent transient inactivator of the enzyme, where the relatively slow O_2-dependent reactivation can be readily demonstrated by enzyme assay of incubations conducted in the absence and presence of O_2. Higher concentrations of methylhydrazine and 1,1-dimethylhydrazine also lead to enzyme inactivation, but the O_2 dependence of reactivation cannot be demonstrated in the same manner since the anaerobically inactivated enzyme recovers instantaneously upon dilution into the (aerobic) assay buffer. Model studies suggest that the unique inactivation effected by hydrazine involves reductive deoxygenation of the TPQ cofactor at C-5, and that the resulting cofactor resorcinol derivative can be re-oxygenated to the active cofactor, both reactions we propose to be facilitated by copper at the BPAO active site.

Results and Discussion

Interaction of BPAO with Hydrazines. (a) With hydrazine. The time courses of activity of BPAO (1.8 μM) incubated with various concentrations of hydrazine at 30 °C under normal aerobic conditions are shown in Figure 1. As shown, at all of the concentrations measured, there was a rapid loss of enzyme activity over a 5 min or shorter time range. The fact that the enzyme was inhibited by nearly 90% by 2.0 μM hydrazine but only partially by 1.0 μM hydrazine is consistent with a bimolecular stoichiometric derivatization of BPAO by hydrazine.[7] In eace case, after the initial loss of activity, the inhibited BPAO began to show recovery of activity within an additional 10 min for concentrations of hydrazine of 2 μM or less (Figure 1), and after a long time (17−19 h), the activity was within 10% of the control activity (not shown). The rate of recovery of enzyme activity following the initial 87% inhibition by 2 μM hydrazine was found to follow first-order kinetics (Figure 1 inset), consistent with an earlier report.[7] At 5 μM, however, no sign of recovery of enzyme activity was seen up to 120 min.

Under anaerobic conditions (argon bubbling), exposure of BPAO to 2 μM hydrazine resulted in the same rapid loss of activity as found under aerobic conditions, but subsequent recovery of activity was not observed. In the presence of a glucose/glucose oxidase (+ catalase) "internal O_2-scrubber" system, no activity recovery was seen in the nearly 300 min that the experiment was allowed to run (Figure 2). The difference is clearly due to O_2: following maintenance of anaerobic conditions for 100 min, exposure of the incubation mixture to air resulted

(10) For an example, see: Wang, F.; Venkataraman, B.; Klein, M. E.; Sayre, L. M. *J. Org. Chem.* **1992**, *54*, 6687−6689. See correction: Wang, F.; Venkataraman, B.; Klein, M. E.; Sayre, L. M. *J. Org. Chem.* **2001**, *66*, 2186.

(11) (a) Mure, M.; Nii, K.; Inoue, T.; Itoh, S.; Ohshiro, Y. *J. Chem. Soc., Perkin Trans. 2* **1990**, 315−320. (b) Mure, M.; Itoh, S.; Ohshiro, Y. *Tetrahedron Lett.* **1989**, *30*, 6875−6878.

(12) Duine, J. A.; Frank, J.; Verwiel, P. E. J. *Eur. J. Biochem.* **1981**, *118*, 395−399.

(13) The balance between hydrazone/azo derivatization and reduction of PQQ models can shift depending on the particular PQQ model employed: (a) Itoh, S.; Fukui, Y.; Ogino, M.; Haranou, S.; Komatsu, M.; Ohshiro, Y. *J. Org. Chem.* **1992**, *57*, 2788−2793. (b) Itoh, S.; Fukui, Y.; Haranou, S.; Ogino, M.; Komatsu, M.; Ohshiro, Y. *J. Org. Chem.* **1992**, *57*, 4452−4457.

(14) Sleath, P. R.; Noar, J. B.; Eberlein, G. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 3328−3338.

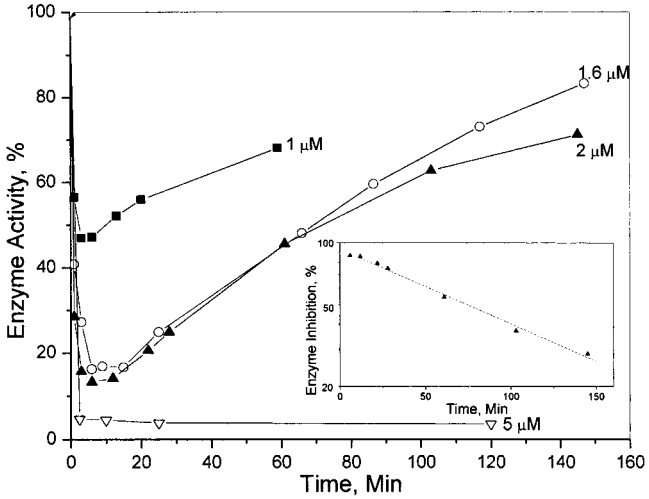


Figure 1. Inactivation of BPAO (1.8 μM) by various concentrations of hydrazine. At various times, aliquots were diluted with excess benzylamine, and the activity relative to the control solution was determined spectrometrically by measurement of the linear production of benzaldehyde for 1 min. The inset shows a semilog plot of the recovery of activity following enzyme inactivation by 2 μM hydrazine.

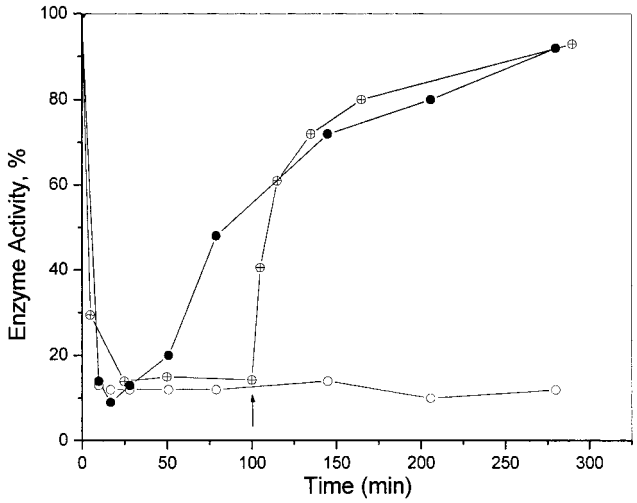


Figure 2. Inactivation of BPAO (1.7 μM) by hydrazine (2 μM) under different incubation conditions: ●, under aerobic conditions; ○, under anaerobic conditions; ⊕, under anaerobic condition for 100 min, then exposure to air (arrow).

in relatively rapid recovery of activity ($t_{1/2} \sim 5$ min) up to the point where the activity joined the progress of the normal aerobic recovery curve (Figure 2). The same behavior was found when the enzyme was initially ~50% inactivated anaerobically (argon bubbling) by 0.8 μM hydrazine, and then O_2 was admitted after 60 min (not shown). It should be noted that O_2-dependent recovery, although rapid, is not instantaneous, because if it were, then aliquoting the anaerobically inhibited enzyme into the air-equilibrated buffered benzylamine assay solution would have resulted in an increasing slope of benzaldehyde production during the 1 min assay (which was not observed).

(b) With Methylhydrazine. The time courses of activity of BPAO when incubated with 4 μM methylhydrazine under aerobic and anaerobic conditions are shown in Figure 3. Under aerobic conditions, there was

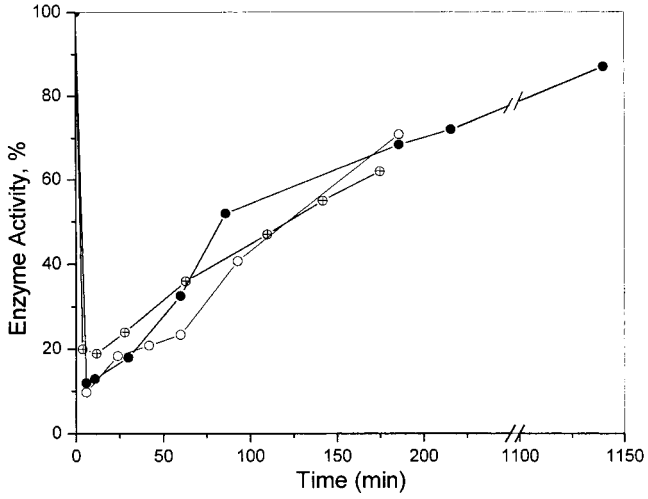


Figure 3. Inactivation of BPAO (1.5 μM) by methylhydrazine (4 μM) under different incubation conditions: ●, under aerobic conditions; ○, under anaerobic conditions (argon bubbling); ⊕, under anaerobic conditions in the presence of glucose/glucose oxidase/catalase.

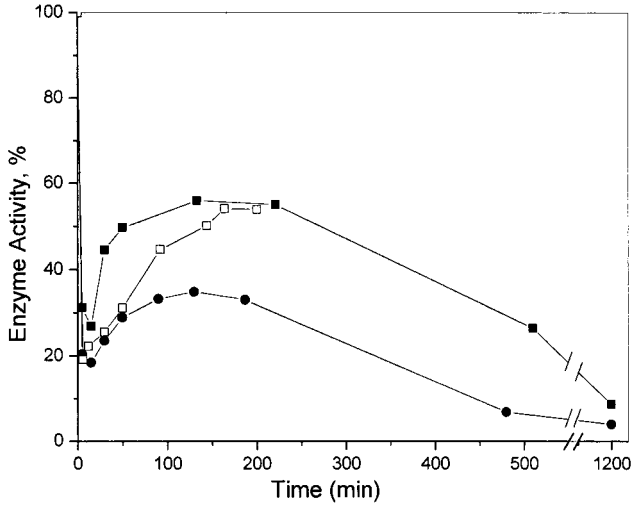


Figure 4. Inactivation of BPAO (1.5 μM) by 1,1-dimethylhydrazine: ■, 40 μM 1,1-dimethylhydrazine under aerobic conditions; □, 40 μM 1,1-dimethylhydrazine under anaerobic conditions; ●, 100 μM 1,1-dimethylhydrazine under aerobic conditions.

observed a rapid >80% loss of activity (the first ~5 min), followed by a recovery that reached 72% of the control activity after 3 h and nearly 90% of the control activity after 19 h. However, unlike the case of hydrazine, essentially the same profile of loss and regain of enzyme activity was observed under anaerobic conditions (argon bubbling), and even under anaerobic conditions using the O_2-scrubber system (Figure 3). Although it appears that the recovery of activity may not be O_2-dependent, we cannot rule out an O_2-dependent recovery that occurs instantaneously upon dilution of the primary incubation mixture into the aerobic assay buffer.

(c) With 1,1-Dimethylhydrazine. The time courses of activity of BPAO when incubated with either 40 or 100 μM 1,1-dimethylhydrazine are shown in Figure 4. The first observation is that substantially higher concentrations, relative to those used for the other hydrazine derivatives, were needed to effect the same level of rapid

enzyme activity loss. Again, after about 10 min, the activity began to recover. As in the case of methylhydrazine, incubation with 1,1-dimethylhydrazine under anaerobic conditions (argon bubbling) also exhibited recovery after the 10-min period of initial activity loss, though slightly slower than was observed under aerobic conditions (Figure 4, compare at 40 μM). The use of an O_2-scrubber system mirrored the argon bubbling results (data not shown). A second observation, however, is that for 1,1-dimethylhydrazine the recovery of enzyme activity under aerobic conditions (at either 40 or 100 μM) was incomplete and began to decrease again after ~150 min. The incompleteness of recovery was more pronounced using 100 μM as opposed to 40 μM 1,1-dimethylhydrazine (Figure 4). For either concentration, the remaining enzyme activity was less than 10% when measured after 20 h relative to the 20 h control sample, suggesting that 1,1-dimethylhydrazine is metabolized to a product that induces true irreversible inactivation of the enzyme. During the same 20 h period, the control incubation mixture lacking inhibitor displayed no detectable deterioration of activity.

Comparing the results for all three hydrazines, we can conclude a relative inhibitory potency order of H_2NNH_2 > $MeNHNH_2$ > Me_2NNH_2 for the initial temporary loss of activity, based on the concentration needed to effect ~90% inhibition. In all three cases, at the point of maximal temporary loss of activity under aerobic conditions, the enzyme was also assayed over time following its isolation by rapid gel chromatography; no significant differences were observed (data not shown) from the course of recovery described in Figures 1, 3, and 4, indicating that maintenance of inhibition does not depend on diffusible species present in solution. One conceivable explanation for the temporary loss of activity induced by hydrazines is that they form reversible, but tight-binding inhibitory complexes with the enzyme, which eventually undergo metabolic turnover to noninhibitory species. Recovery of BPAO activity is not due to O_2-dependent oxidation of the equilibrium amount of free hydrazines present in solution. This was demonstrated by showing that there was no detectable O_2 consumption (in 90 min) when each of the three hydrazines (up to 100 μM) was incubated in the absence of enzyme. In addition, if the buffered hydrazine solutions were kept at 30 °C for 2 h prior to the addition of enzyme, the identical reaction courses were observed as described above (data not shown), showing that the hydrazines do not decompose nonenzymatically under the reaction conditions.[15]

On the other hand, in the presence of enzyme, all three hydrazines induced O_2 consumption. Using 0.67 μM enzyme and all the hydrazines at the same concentration (20−100 μM), the rank order of O_2 uptake was Me_2NNH_2 > $MeNHNH_2$, H_2NNH_2, reflecting the fact that in this concentration range the enzyme is least inhibited by 1,1-dimethylhydrazine and thus more capable of turnover. We did not attempt to reconcile the stoichiometry of O_2 uptake with the level of inhibition seen, because of the complicated balance between metabolic turnover of hydrazine compound and the onset/decay of inhibition and because of the complication of the slow-onset irreversible inactivation induced by 1,1-dimethylhydrazine. Nonetheless, the data clearly indicate that BPAO carries out an O_2-dependent metabolism of all three hydrazines, *consistent with the recovery of enzyme activity under aerobic conditions reflecting the point of irreversible metabolic consumption of all added hydrazine compound at the concentration used*.

It is clear that the temporary inactivation process effected by hydrazine is different from that effected by methylhydrazine or 1,1-dimethylhydrazine in that only the former exhibits a directly observable O_2 dependence for reactivation. In addition, 1,1-dimethylhydrazine is distinguished in exhibiting an apparent irreversible inactivation effect at long incubation times. We believed that model reactions of a suitable TPQ analogue with these hydrazines would provide important clues as to the nature of the reaction intermediates or products that are ultimately required to explain the mechanism of transient inhibition. In addition, model TPQ−hydrazine experiments were of interest in their own right in terms of how these relate to model reactions of TPQ with primary amine substrates.[16,17]

Reaction of TPQ Model 1 (TBHBQ) with Hydrazine. (a) Deoxygenation To Give Resorcinol 5. An NMR tube-scale reaction of the TPQ model 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone (TBHBQ, **1**) with hydrazine (8 equiv) in CD_3CN was monitored by ^1H NMR spectroscopy over a period of 1 week. The earliest spectrum recorded (2 h) showed that the starting TBHBQ had disappeared, and a mixture of hydrazone **3**, triol **2**, and resorcinol **5** (Scheme 3) was identified in a ratio of 10:2:1 by integration of the corresponding signals. The product mixture changed after 24 h, in that resorcinol **5** became a major component in excess of the amount of hydrazone **3**, whereas triol **2** remained as a minor product. After 7 days at room temperature, resorcinol **5** was observed as the sole product.[18] Assignment of hydrazone **3** rather than azo tautomer **4** as the observed intermediate was based on the quinone-like chemical shifts for the two vinyl signals.

The same reaction of TBHBQ with hydrazine (8 equiv) in degassed DMSO-d_6 followed by ^1H NMR spectroscopy displayed a similar distribution of products, except the progression of reaction was slower in this solvent. After 20 min, hydrazone **3** and triol **2** were seen in a ratio of 6:1 along with a trace amount of resorcinol **5**. Over several days, signals corresponding to **5** increased with concomitant decrease of hydrazone **3**. After 5 weeks the spectrum revealed a mixture of hydrazone **3**, triol **2**, and resorcinol **5** in a ratio of 2:1:12.

Taken together, the results in both solvents suggest that the TBHBQ reaction with hydrazine leads initially to the hydrazinocarbinol intermediate **6**, which partitions between (reversible) dehydration to give hydrazone **3** (major pathway) and irreversible elimination of diimide to give triol **2** (minor pathway), analogous to what was proposed in PQQ model reactions.[11,14] It is interesting

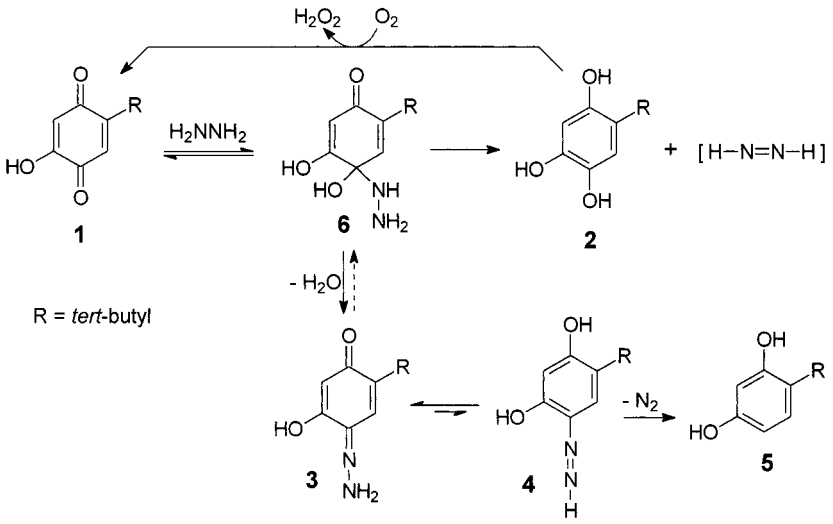
(15) This absence of spontaneous decay was also noted previously for β-hydroxyethylhydrazine.[7]

(16) (a) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 3096−3105. (b) Lee, Y.; Sayre, L. M. *J. Am. Chem. Soc.* **1995**, *117*, 11823−11828.

(17) (a) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8698−8706. (b) Mure, M.; Klinman, J. P. *J. Am. Chem. Soc.* **1995**, *117*, 8707−8718.

(18) The disappearance of triol **2** in CD_3CN, ultimately leaving resorcinol **5** as a single product, is likely due to oxidative recycling of **2** to **1** by O_2 dissolved in the solvent. This interpretation is supported by the finding that when the same reaction was carried out in CD_3CN free of O_2 in a carefully sealed tube, triol **2** was still observed even at the point where all hydrazone **3** was converted to **5**.

Scheme 3



that although the dehydration pathway is heavily favored, the elimination reaction does compete in the hydrazine case, in contrast to reactions of TBHBQ with *amines*, where the initial carbinolamines undergo exclusive dehydration (to quinoneimines or iminiums).[15b] This difference may have its roots in either kinetic or thermodynamic biases between expulsion of a N=N and a C=N species. Following the initial disappearance of starting TBHBQ, the observed changes in the product composition with time reflect the ultimate irreversible conversion of hydrazone **3** (initially major, becoming minor) to resorcinol **5**.[18]

The tautomeric azo form **4** of hydrazone **3**, although not detected in our reactions, is believed to be the putative intermediate leading to the resorcinol product (Scheme 3). Monosubstituted azo compounds (ArN=NH or RN=NH) are known to be unstable, eluding isolation, unlike their N,N′-disubstituted counterparts. Decomposition of PhN=NH, giving N_2 and benzene, is proposed to involve a free-radical mechanism[19,20] and is known to be accelerated by redox-active transition metal ions.[19] In fact, the very slow conversion of hydrazone **3** to resorcinol **5** was accelerated by addition of a catalytic amount of Cu(II) to the reaction mixture. Addition of Cu(II) to the reaction of TBHBQ (**1**) and hydrazine (8 equiv) in CD_3CN, at the point where only a trace of resorcinol **5** was seen by spectral monitoring, induced instant bubbling of gas (undoubtedly N_2) from the solution. Recording the ^1H NMR spectrum 15 min after the addition of Cu(II) revealed that the major hydrazone **3** component of the reaction had been cleanly converted to resorcinol **5,** leaving triol **2** as the only minor species. Although the effect of Cu(II) is believed to be mainly that of facilitating decomposition of azo species **4** in equilibrium with hydrazone **3**, we cannot exclude the possible catalysis of the latter equilibration as well.

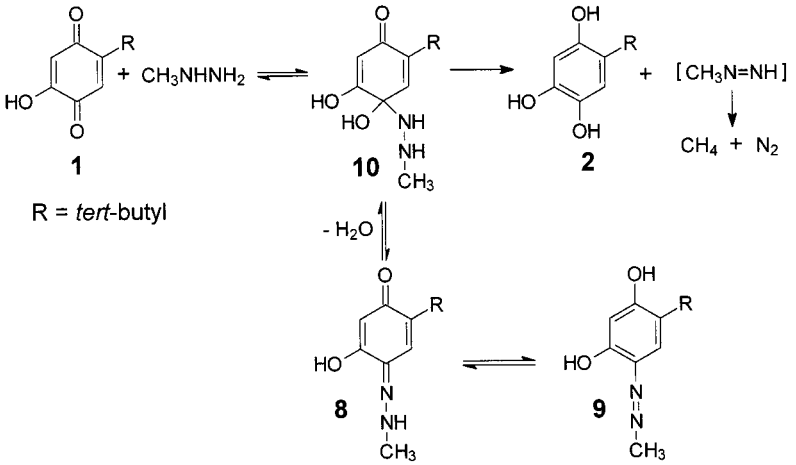
As discussed above, the finding that inactivation of BPAO by hydrazine is recoverable upon exposure to O_2 (Figure 1) implicates conversion of the cofactor to a form that can be reconverted to the active quinone form by O_2 concomitant with the consumption of hydrazine. One possibility is that inhibition reflects tying up of the cofactor as hydrazone **3** (Scheme 3, R = protein), with slow equilibrating hydration to **6**, which eventually is "funneled" off by elimination of diiimide to give the reduced triol form **2** (TOPA) of the cofactor. Since the TOPA form of the enzyme should readily reoxidize to the native quinone form (TPQ), this scenario would rationalize aerobic recovery of enzyme activity. However, if eventual reduction of **3** to TOPA were responsible for recovery of activity, this irreversible transition would occur over a time course that is independent of O_2. Thus, if one were to assay an aliquot of the anaerobic incubation mixture at a time point when the aerobically inhibited enzyme had fully recovered (e.g., >200 min, Figure 2), one would expect that the TOPA form of the enzyme present at that time would immediately regain activity in the aerobic benzylamine assay mix that would become apparent *during the 1 min duration of the assay*. This was not observed.

Another possibility (see Scheme 3) is that the eventual recovery of enzyme activity represents slow irreversible conversion of hydrazone **3** (Scheme 3, R = protein) via its azo tautomer **4** into resorcinol **5** and N_2, a reaction that represents net formal deoxygenation of the cofactor, and could well be catalyzed by the active-site copper. According to this scenario, if the resorcinol could undergo an O_2-dependent reoxygenation to regenerate the native TPQ form of the cofactor, this scenario would also explain the recovery of enzyme activity over time.

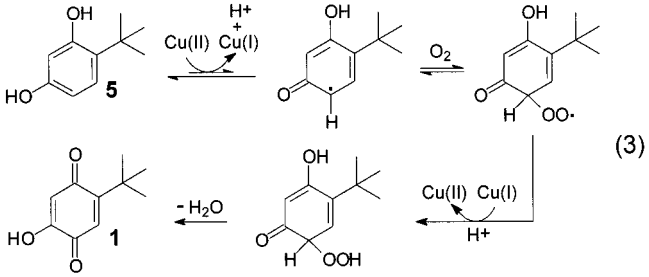
(b) Cu(II)-Mediated Oxygenation of Resorcinol 5 To Give TBHBQ (1). Focusing therefore on the possibility of re-oxygenation of an active-site resorcinol, oxidation of 4-*tert*-butylresorcinol (**5**) was investigated by monitoring its reaction in aqueous acetonitrile (1:1) in the presence and absence of a catalytic amount of Cu(II) at various pH settings (7−11) under aerobic conditions. Whereas starting resorcinol **5** was recovered from the reaction without Cu(II), the reaction conducted in the presence of Cu(II) afforded as major products a 3:1 mixture of TBHBQ **1** and a byproduct representing further oxidation of **1** in the presence of oxygenated **5**.[21] Demonstration of the facile generation of TPQ model **1** from resorcinol **5** under mild conditions aided by Cu(II)

(19) Huang, P. C.; Kosower, E. M. *J. Am. Chem. Soc.* **1968**, *90*, 2367−2376.
(20) Kosower, E. M.; Huang, P. C.; Tsuji, T. *J. Am. Chem. Soc.* **1969**, *91*, 2325−2329.

Scheme 4



(a possible mechanism is shown in eq 3)[22] is consistent with the deoxygenation/reoxygenation sequence shown in Scheme 3 as the rationale for the temporary inactivation of BPAO by hydrazine.



Reaction of TBHBQ (1) with Methylhydrazine and 1,1-Dimethylhydrazine in CD_3CN. The reaction of the TBHBQ with excess methylhydrazine (3−10 equiv) in CD_3CN was monitored by ^1H NMR spectroscopy. The reaction quickly (30 min) exhibited a spectrum consistent with azo derivative **9**, and little change occurred over several days. Assignment of the derivative as azo **9** rather than hydrazone tautomer **8** was based on its displaying widely divergent vinyl chemical shifts expected of a resorcinol. The same reaction performed in degassed CD_3CN using 2 equiv of methylhydrazine and 2 equiv of *tert*-butylamine (added as a base catalyst) revealed azo derivative **9** and triol **2** in a ratio of 4:1 in the ^1H NMR spectrum after 20 min, and no change was noticed for a 2-week period. It appears that the major azo-forming pathway is accompanied by a minor elimination pathway that gives rise to triol **2** and the metastable species $CH_3N{=}NH$, which decomposes to N_2 and methane (Scheme 4), as indicated by observation of a singlet at δ 0.2 ppm in the ^1H NMR spectrum that disappeared upon a freeze−pump−thaw degassing cycle. This signal was verified as CH_4 by direct bubbling of the latter through CH_3CN. The lack of appearance of triol **2** in the first experiment (without degassing) presumably reflects a reoxidative recycling of **2** to **1**.

When the reaction of TBHBQ with excess 1,1-dimethylhydrazine (or 2 equiv of 1,1-dimethylhydrazine and 2 equiv of *tert*-butylamine) in degassed CD_3CN was monitored by ^1H NMR spectroscopy, several new species were found in the earliest spectrum recorded (5 min). One major component of the mixture was identified as triol **2**. Surprisingly, the second major component of the mixture was the same azo derivative **9** found in the reaction of TPQ model **1** with methylhydrazine. The signals corresponding to both triol and azo derivatives increased as the starting quinone **1** was consumed.

However, once **1** had completely disappeared, signals corresponding to the two initial major products also decreased with time over the course of 2 days, to be replaced by a new major product. On the basis of the observed ^1H NMR signals, this compound was identified as hydroxymethylated azo derivative **11** or its hydrazone tautomer (Scheme 5), the product of an electrophilic aromatic substitution reaction of azo derivative **9** with formaldehyde,[24] apparently derived from one methyl group of 1,1-dimethylhydrazine. Moreover, after 5 days, and brief exposure to air (to autoxidatively recycle remaining triol **2**), diaryl product **12** crystallized from the NMR tube and was fully characterized. The hydrazone rather than azo tautomer has been assigned to this structure on the basis that the CH_3 6H signal is a doublet and the appearance of a 2H signal at δ 12.1 assigned to the NH signal (if this signal represented OH, it should integrate for 4H). Compound **12** represents a second electrophilic substitution of **9** with **11**. At no point in the reaction was there appearance of the upfield singlet characteristic of CH_4, in contrast to the reaction with methylhydrazine.

Justification of the demethylation mechanism is as follows (Scheme 5). The only possible hydrazone/azo tautomerization for species **14**, arising from 1,1-dimethylhydrazine via hydrazinocarbinol **13**, would require (probably unfavorable) equilibration with the cationic azo species **15** that, in turn, would be in equilibrium with iminium species **16**. Hydrolytic loss of formaldehyde from the iminium tautomer **16** to give arylhydrazine **17** would drive the unimolecular equilibria from **14** to **16**. Oxidation of **17** by starting quinone **1** would generate azo

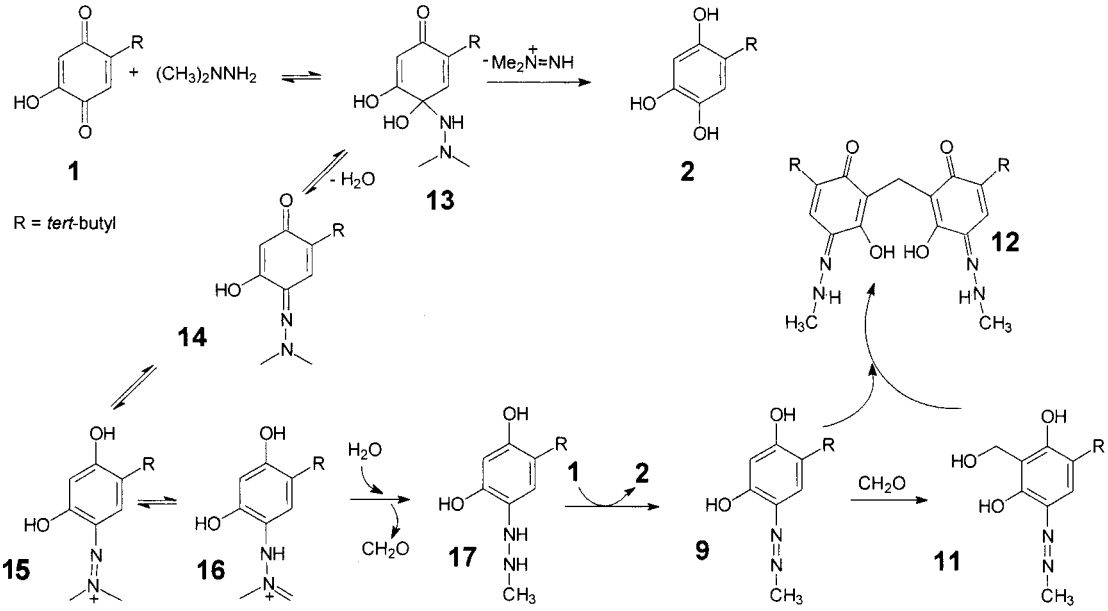
(21) The byproduct formed red crystals in the NMR tube (CD_3CN). Its identity was shown to be 3-(4-(1,1-dimethylethyl)-5-hydroxy-2-oxo-(5*H*)-furan-5-yl)-5-hydroxybenzofuran-2,6-dione by an X-ray diffraction study. Structural details and the proposed mechanism of its formation will be described elsewhere: Lee, Y.; Macikenas, D.; Protasiewicz, J. D.; Sayre, L. M. Manuscript in preparation.

(22) Copper(II)-mediated oxygenation of 5-alkylresorcinols to 2-hydroxy-1,4-benzoquinones has been reported,[23] though our report is the first to our knowledge for a 4-alkylresorcinol.

(23) (a) Singh, U. S.; Scannell, R. T.; An, H.; Carter, B. J.; Hecht, S. M. *J. Am. Chem. Soc.* **1995**, *117*, 12691−12699. (b) Starck, S. R.; Deng, J.-Z.; Hecht, S. M. *Biochemistry* **2000**, *39*, 2413−2419.

(24) For a review, see: Schnell, H.; Krimm, H. *Angew. Chem., Int. Ed. Engl.* **1963**, *2*, 373−379.

Scheme 5



compound **9** and triol **2**. Alternatively, triol **2** could additionally arise from irreversible elimination of the unstable species $(CH_3)_2N^+{=}NH$ from the hydrazinocarbinol **15**, though no independent evidence for this step was apparent. Presumably the two major products found at the early stage of reaction, triol **2** and azo derivative **9**, disappeared at the end of reaction due to oxidative recycling of the former and condensation of the latter with formaldehyde to give **11** and then **12**. It is noteworthy that resorcinol **5** was not observed in the reactions of **1** with either methylhydrazine or 1,1-dimethylhydrazine.

Reaction of TBHBQ (1) with Methylhydrazine and 1,1-Dimethylhydrazine in DMSO-d_6. In studies on the reaction of TPQ model **1** with benzylamine, we demonstrated that solvent changes could alter the reaction course by changing the lifetime of various reaction intermediates.[16b] For example, a large deuterium kinetic isotope effect was observed for the reaction of **1** with d_0- and d_2-benzylamine in CD_3CN, but not in DMSO-d_6. In the anaerobic reaction of TBHBQ with methylhydrazine (2−8 equiv) in degassed DMSO-d_6, the earliest ^{1}H NMR spectrum (15 min) displayed an equal amount of triol **2** and what appeared to be either azo compound **9** or the hydrazone tautomer **8** (with upfield vinyl signals compared to **9**), depending on the molar excess methylhydrazine used. Unlike the case in CD_3CN, where azo derivative **9** survived for 2 weeks without any decomposition, hydrazone/azo **8**/**9** was cleanly converted to triol **2** when the reaction tube was allowed to stand overnight. An additional signal that grew at δ 0.2 during this time was attributed to methane. Conversion of **8**/**9** to **2** presumably occurs by reversible hydration of **8** followed by elimination of $CH_3N{=}NH$ (Scheme 4). The different reaction course in DMSO-d_6 compared to CD_3CN probably reflects the effect of the solvent change on the hydrazone−azo equilibrium, which is known to be sensitive to medium effects.[25]

The anaerobic reaction of TBHBQ with 1,1-dimethylhydrazine (2−8 equiv) in degassed DMSO-d_6 led to the same hydrazone/azo **8**/**9** species observed for methylhydrazine much more quickly than in CD_3CN, along with less triol **2** (and a minor unidentified product). This finding indicated that the hydrazone-forming reaction with irreversible release of formaldehyde (Scheme 5) was fast in this case relative to competing reactions, though the oxidation of **17** by **1** needed to generate hydrazone **8** should have produced an equimolar amount of **2**. The minor unidentified species could be derived from **2** and the released formaldehyde. Hydrazone/azo **8**/**9** disappeared with time and was converted to triol **2** when the reaction tube was allowed to stand overnight. Overall, the reaction in DMSO-d_6 can be summarized as following Scheme 5 up to compound **9** and from there, Scheme 4.

Mechanism of Inactivation of BPAO by Methylhydrazine: Anaerobic Enzyme Behavior and Product Study on the Inactivation of BPAO by 3-Phenylpropylhydrazine. The model studies using methylhydrazine suggest a very different mechanism for reactivation of BPAO than in the case of hydrazine itself. Spectroscopic studies reported previously[6,9] support a hydrazone rather than azo tautomer for the enzyme derivative (eq 2). According to Scheme 4, generation of the hydrazone derivative **8** via hydrazinocarbinol **10** would represent the inactive form of the enzyme, in competition with a minor (unnoticeable) pathway involving elimination of $CH_3N{=}NH$ from **10**. Over time, however, the hydrazone, in equilibrium with **10**, albeit unfavorable, would slowly be drained off by elimination of $CH_3N{=}NH$ from **10** to give the triol form of the cofactor (TOPA). Although **10** could also dissociate to regenerate active enzyme, only the elimination pathway would correspond to metabolism of methylhydrazine, thus allowing for the permanent recovery of activity. The TOPA form of the enzyme, when generated, would immediately oxidize to the catalytically active quinone form (TPQ) upon exposure to O_2, a reaction that is presumed to be catalyzed by the enzyme.[26]

Assuming that this reoxidation would occur instantaneously when the methylhydrazine-inactivated enzyme

(25) Doroshina, G. P.; Kotov, A. V. *Zh. Obshch. Khim.* **1980**, *50*, 1635−1638.

(26) Nakamura, N.; Matsuzaki, R.; Choi, Y.-H.; Tanizawa, K.; Sanders-Loehr, J. *J. Biol. Chem.* **1996**, *271*, 4718−4724.

is diluted into the air-equilibrated assay buffer, it would not be possible to determine by activity assay that the enzyme is in the TOPA rather than TPQ form. This would explain why the time course for recovery of activity was the same whether the reaction was run aerobically or anaerobically. Namely, the various points along the activity vs time recovery curve (Figure 3) represent that fraction of the enzyme for which the hydrazone derivative has been converted (Scheme 4) to the TOPA form. Under aerobic conditions, this fractional TOPA form is reoxidized to TPQ, whereas under anaerobic conditions, this same enzyme fraction remains in the TOPA form until the incubation aliquot is diluted into the assay buffer.

Since we could thus not demonstrate the O_2 dependence of activity recovery by activity assay of aerobic vs anaerobic incubations, we sought an independent approach to confirm that the time course of activity recovery actually did reflect the conversion of the methylhydrazone **8** to the TOPA form as proposed. In the anaerobic incubation, at the time points corresponding to recovery of activity (e.g., 40−50% at 100 min, Figure 3), if the enzyme were in the TPQ form, then addition of phenylhydrazine should result in appearance of the 450 nm derivative band with an intensity reflecting the fraction of active enzyme. On the other hand, if this same fraction of enzyme were in the TOPA form, there would be no spectral change upon addition of phenylhydrazine. Under the conditions of Figure 3 (1.5 μM BPAO, 4 μM $MeNHNH_2$) excess phenylhydrazine (16 μM) was added to both aerobic and anaerobic incubations at two time points, 3 and 100 min. At 3 min, the enzyme is present almost totally (80−90%, Figure 3) as the methylhydrazone, and there was no spectral change apparent upon addition of phenylhydrazine for either incubation (not shown). This indicates that phenylhydrazine is not capable of displacing methylhydrazine from the TPQ methylhydrazone. In contrast, at 100 min (Figure 3 indicates 40−50% activity), although addition of phenylhydrazine to the aerobic incubation produced about half the increase in A_{450} found for the fully active control enzyme, *addition of phenylhydrazine to the anaerobic incubation produced no spectral change* (not shown). This result confirms that recovery of enzyme activity in the metabolism-dependent inactivation by methylhydrazine ultimately reflects O_2-dependent oxidation of TOPA to TPQ.

The proposal that model-study-derived Scheme 4 also represents the enzyme mechanistic course additionally hinges on whether the elimination of $CH_3N{=}NH$ from the hydrazinocarbinol, evident in the model study by observation (^{1}H NMR) of CH_4, actually occurs in the case of the enzyme. In the enzyme case, trapping and detection of the small amount of CH_4 that would be generated from metabolism of methylhydrazine would be a formidable task, and we thus queried that if other monoalkylhydrazines behaved like methylhydrazine, we could then investigate the detection of less elusive $RN{=}NH$ decomposition products. In this regard, we prepared 3-phenylpropylhydrazine by the substitution reaction of 3-phenylpropyl bromide with hydrazine. The time courses of inactivation of BPAO by various concentrations of 3-phenylpropylhydrazine are shown in Figure 5. Analogous to what was found with methylhydrazine, the enzyme activity fell to zero in less than 5 min at all of the concentrations measured, but then recovered after approximately 25−175 min depending on the initial concentration (5−25 μM) of 3-phenylpropylhydrazine used, reflecting the amount of starting hydrazine that needed to be metabolized. At 50 μM, enzyme activity exhibited no recovery up to 660 min, but after 23 h, the activity recovered to 53% (not shown in Figure 5).

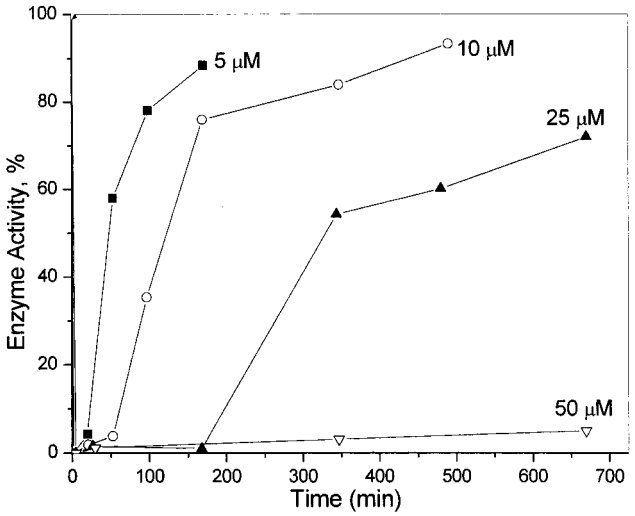


Figure 5. Inactivation of BPAO (1.3 μM) by various concentrations of 3-phenylpropylhydrazine.

To identify the product of 3-phenylpropylhydrazine metabolism by BPAO, a large-scale incubation with excess 3-phenylpropylhydrazine was conducted at 30 °C for 20 h, and the solution was then extracted with ether. The residue obtained following drying and evaporation of the organic layer was analyzed by GC-MS and found to be propylbenzene, as indicated by its exhibiting the same retention time, same molecular ion peak (*m*/*z* 119), and same fragmentation peaks with authentic propylbenzene. As a control, the identical incubation reaction run in the absence of BPAO failed to reveal any significant organic product detectable by GC-MS. Taken together, these experiments confirm the mechanistic proposal for methylhydrazine inactivation/reactivation made on the basis of the model studies (Scheme 4).

Enzyme Studies of BPAO with Formaldehyde. The finding that interaction of BPAO with 1,1-dimethylhydrazine is unique in that the initial activity loss is followed by only partial recovery and then a slow subsequent irreversible loss of activity (Figure 4) led us to consider that the latter loss might reflect metabolic release of formaldehyde at the active site and modification of the cofactor, as occurred in the model study with TBHBQ in CD_3CN. Release of formaldehyde was confirmed by isolation of its 2,4-dinitrophenylhydrazone by quenching the enzyme reaction with acidic (HCl) 2,4-dinitrophenylhydrazine solution. Therefore, enzyme studies were carried out to determine whether formaldehyde itself might inactivate the enzyme at concentrations consistent with those that would form during turnover. Formaldehyde was found to be a time- and concentration-dependent inactivator of BPAO (Figure 6), though the degrees of inhibition found using 40 μM $HCH{=}O$ at 500 min and 20 h were less than the decreases in activity found using 40 μM 1,1-dimethylhydrazine at these times (Figure 4) from the point of the extrapolated recovery curve. Using 100 μM formaldehyde, the level of inhibition found was again less than that predicted from extrapola-

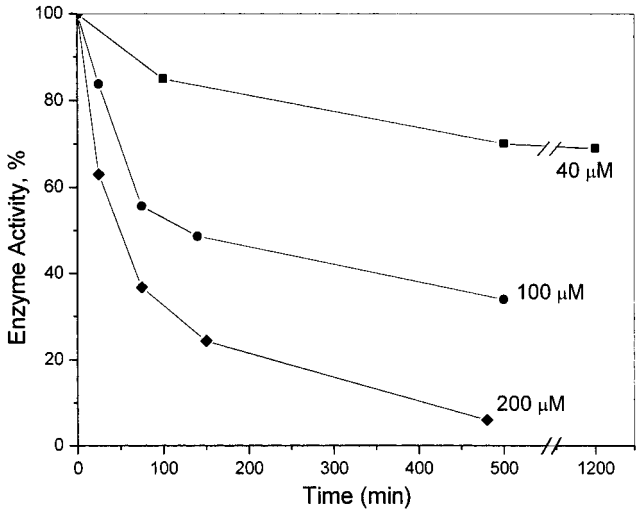


Figure 6. Inactivation of BPAO (1.3 μM) by various concentrations of formaldehyde.

tion of the recovery curve for 100 μM 1,1-dimethylhydrazine (Figure 4). These results suggest that metabolically released formaldehyde could contribute to a major portion of the nonrecoverable inactivation by 1,1-dimethylhydrazine found at long reaction times, though other factors must also be involved.

Conclusions. Mechanism of Temporary Inactivation of BPAO by Hydrazine, Methylhydrazine, and 1,1-Dimethylhydrazine. The interaction of BPAO with all three hydrazines results in a rapid loss of activity that, however, eventually recovers, indicative of metabolism of each added hydrazine compound. At the minimal concentration of hydrazine compound needed to achieve ~90% inhibition, this recovery begins within the subsequent 10 min of incubation, though longer times are needed when higher concentrations of hydrazine compound are used (more time is needed for complete metabolism). At the point of maximal activity loss, gel filtration does not restore activity, nor does it modify the recovery phase, suggesting that the inactive enzyme represents a covalent or tight-binding derivative, consistent with previously obtained radiolabeling evidence[7] and spectroscopic evidence (obtained rapidly) for a derivatized TPQ cofactor.[6,9] Solution studies using the TPQ model TBHBQ (**1**) provide clues as to the nature of the inactive species involved in each case as well as the mechanism responsible for recovery of activity. For all three hydrazines, metabolic turnover is an O_2-dependent process; this could be demonstrated by direct activity assays of aerobic vs anaerobic incubations in the case of hydrazine itself, but a spectroscopic tactic was required in the case of methylhydrazine.

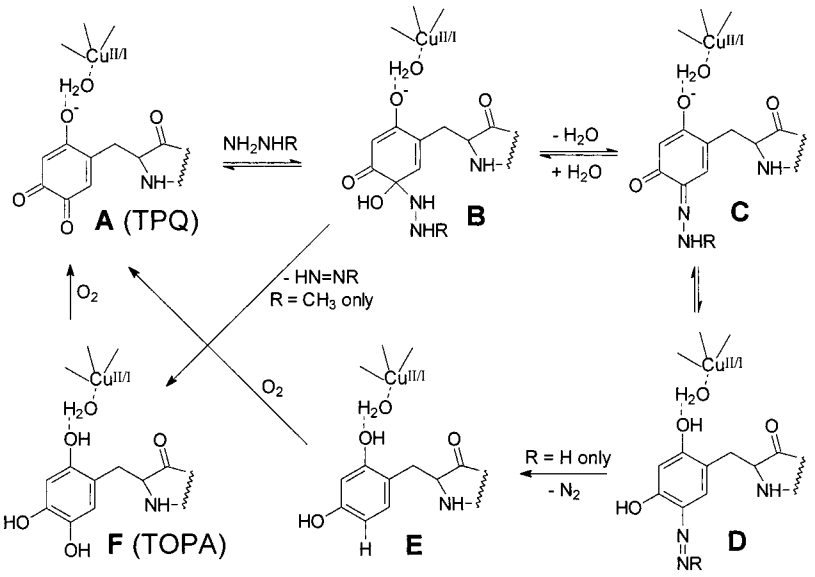
Model studies indicate that for hydrazine and methylhydrazine, dehydration of the initial hydrazinocarbinol intermediate to give hydrazone/azo derivative is favored over the competing irreversible 1,2-elimination to give triol and RN=NH (R = H or CH_3). The reaction of TPQ model **1** with 1,1-dimethylhydrazine leads rapidly to the same hydrazone/azo species generated from methylhydrazine, implicating loss of one of the two methyl groups as the source of the formaldehyde generated. Most interestingly, in the case of hydrazine, the major product besides hydrazone was the C5-deoxygenated (resorcinol) form of the TPQ model **1**.

Our proposed explanation for the enzyme behavior based on the combination of evidence from previous[6,7,9] and current studies is shown in Scheme 6. In the case of methylhydrazine, the initially formed inactive derivative of the enzyme that survives gel chromatography is hydrazone **C** (R = CH_3). However, **C** remains in (unfavorable) equilibrium with the hydrazinocarbinol **B**, which periodically undergoes irreversible elimination of HN=NCH_3 to give triol **F** (TOPA) rather than dehydration back to **C**. The slow recovery of enzyme activity reflects the inexorable progress of the irreversible shift from **C** to **F**. When the reaction is run aerobically, the reoxidation of **F** to the catalytically active quinone form **A** (TPQ) occurs instantly as it is formed, whereas under anaerobic incubation conditions the TOPA form **F** builds up in solution. However, upon dilution of aliquots of the anaerobic reaction into the aerobic benzylamine assay mixture, that fraction of enzyme present as **F** rather than **C** instantaneously oxidizes to the catalytically active native TPQ state. We propose that reoxidation of the TOPA triol is an enzyme-mediated[26] O_2-dependent process analogous to that utilized in reoxidation of the substrate-amine-derived aminoresorcinol. Thus, reoxidation of the TOPA form by the enzyme would recruit O_2 as effectively as any other enzymatic O_2 "scrubber". Thus, there would be no way to prevent TOPA reoxidation in any *activity assay* designed to show that the enzyme is present in the TOPA rather than TPQ form. Notwithstanding, evidence for the buildup of the TOPA form **F** under anaerobic conditions was shown by the inability of the enzyme to react with phenylhydrazine. Evidence for irreversible elimination of HN=NCH_3 in the enzyme case is the observation of propylbenzene concomitant with recovery of the enzyme initially inactivated by phenylpropylhydrazine. Overall, ultimate recovery of activity in the case of methylhydrazine represents its oxidative metabolism to N_2 and CH_4.

In the case of dimethylhydrazine, the inactive form of the enzyme that survives gel filtration could be the simple dimethylhydrazone analogous to **14** in Scheme 5 (no azo tautomer is possible), though our inability to observe this species in the model study suggests that it undergoes rapid conversion, with release of formaldehyde (through species akin to **15**−**17**) and oxidation, ultimately giving the same hydrazone **C** generated from methylhydrazine. The hydrazone-like spectrum found[9] would not permit a distinction between these two possibilities. Once **C** is formed, regain of activity occurs as in the case of methylhydrazine (Scheme 6). Thus, recovery of activity represents metabolism of 1,1-dimethylhydrazine to CH_2=O, N_2, and CH_4. However, following recovery, a second loss of activity occurs at long reaction time, in part from irreversible modification of the enzyme by the released formaldehyde.

In the case of hydrazine itself, there is the unique option for decomposition of the azo tautomer **D** (R = H) of the spectrally observed[6,9] hydrazone **C** (R = H) to N_2 and the C-5-deoxygenated resorcinol cofactor form **E** of the enzyme, a reaction probably facilitated by the active-site copper and apparently more rapid than the alternative hydration of hydrazone **C** (R = H) and subsequent elimination of HN=NH. Resorcinol **E** cannot be reoxidized to **A** instantly and thus shows up in the benzylamine assay as inactive enzyme when the hydrazine incubation is run anaerobically. When the hydrazine incubation is performed in the presence of O_2, however,

Scheme 6



there is time to achieve reoxygenation of the cofactor, thereby regenerating active enzyme. This interpretation is based on the conclusion that the rate-limiting step for recovery of activity reflects conversion of **C** to resorcinol **E** rather than oxygenation of the latter. This is borne out by the study where O_2 was admitted following 100 min of anerobisis (Figure 2) and the finding that the activity at that point rose rapidly only to where the normal aerobic recovery curve had progressed at that point. The finding in model studies that oxygenation of resorcinol did not occur in the absence of copper(II) suggests that the active-site copper mediates oxygenation of **E**, an aspect which is not surprising in light of the critical role of the active-site copper in mediating oxygenation of the active-site tyrosine in the first step of biogenesis of the TPQ cofactor.[27]

Finally, it would be of interest to confirm the hydrazine-induced deoxygenation of the TPQ cofactor by direct analysis of purified enzyme. One possibility would be to look for incorporation of ^{18}O into the enzyme by its reaction with hydrazine in the presence of $^{18}O_2$. Such studies are planned for future work. The C5-deoxygenation represents a so far unprecedented type of transformation that may be of interest in terms of chemical re-engineering of the active site. In the meantime, it is worth emphasizing the difference in behavior of BPAO toward hydrazine and its alkyl derivatives on one hand and the arylhydrazines on the other hand: unlike the hydrazones formed in the former case, arylhydrazine derivatization leads to tautomeric azo derivatives strongly stabilized by extended conjugation, and there is no evidence for metabolism, consistent with the historical use arylhydrazines for stoichiometric derivatization of the TPQ cofactor.

Experimental Section

General Methods, NMR spectra were obtained at 300 MHz (^1H NMR) or 75 MHz (^{13}C NMR), with chemical shifts being referenced to TMS or the solvent peak. Attached proton test (APT) designations, + (CH_2, C) or − (CH_3, CH), are given for some ^{13}C NMR spectra. High-resolution mass spectra (HRMS) were obtained in either EI or FAB modes. For anaerobic UV−vis and NMR experiments, the solvent was thoroughly degassed by "freeze−pump−thaw" prior to the admission of argon. Thin-layer and preparative-layer chromatography were run on Merck silica gel 60 plates with a 254 nm indicator. All solvents, reagents, and organic fine chemicals were the purest available from commercial sources. 5-*tert*-Butyl-2-hydroxy-1,4-benzoquinone (**1**) and 5-*tert*-butyl-1,2,4-benzenetriol (**2**) were prepared and characterized as in our previous studies.[16] All evaporations were conducted at reduced pressure using a rotary evaporator. Bovine plasma amine oxidase (40−100 units/g of protein) suspension in 2 M ammonium sulfate, pH 6, and PDX G.F. 25 were purchased from Sigma. In some experiments, aliquots of the enzyme were dialyzed against 100 mM pH 7.2 sodium phosphate buffer to remove the ammonia, but this had no effect on the results and thus most of the experiments were conducted with the undialyzed commercial enzyme.

Preparation of 4-*tert*-Butylresorcinol (5). 4-*tert*-Butylresorcinol was synthesized according to standard methods with some modifications. To a solution of resorcinol (20 g, 0.18 mol), *tert*-butyl alcohol (17 mL), and glacial acetic acid (30 mL) was added H_2SO_4 (10 mL) over a period of 10 min with ice bath cooling. The reaction mixture was then allowed to stir at room temperature for 30 min. A solution of saturated NaCl (40 mL) was added with stirring, and the mixture, after diluting with water (150 mL), was extracted with Et_2O (150 mL). The ether layer was washed with 12% aqueous NaOH (100 mL), and the aqueous layer was acidified with 6 N HCl to pH 6. After extraction of the aqueous layer with Et_2O, followed by drying (Na_2SO_4) and concentration of ether layer, pure 4-*tert*-butylresorcinol[17a] **5** (15 g, 50%) was obtained as a solid: ^1H NMR ($CDCl_3$) δ 1.38 (s, 9H), 6.26 (d, J = 2.55 Hz, 1H), 6.36 (dd, J = 8.40, 2.55 Hz, 1H), 7.11 (d, J = 8.40 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 29.9 (3C), 34.0, 104.2, 107.0, 127.8, 129.2, 154.2, 155.4.

Cu(II)-Catalyzed Oxidation of 4-*tert*-Butylresorcinol (5) in Aqueous Acetonitrile. A mixture of 4-*tert*-butylresorcinol (**5**, 166 mg, 1 mmol) and $Cu(ClO_4)_2 \cdot 6H_2O$ (37 mg, 0.1 mmol) in 100 mL of acetonitrile−water (1:1) was adjusted to pH 10.0 by adding 4 N NaOH. The solution was stirred vigorously in an open 250 mL Erlenmeyer flask at 25 °C with monitoring of pH. As the reaction progressed, the pH dropped and was readjusted to pH 10 by addition of 4 N NaOH. After 1.5 h of stirring, the reaction mixture was acidified with HCl to pH 3.0, concentrated to remove the CH_3CN, and extracted with EtOAc (50 mL). The organic layer was dried (Na_2SO_4)

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and evaporated, and the residue was dissolved in CD_3CN. The ^1H NMR spectrum showed a mixture of **1** and a byproduct[21] in a ratio of 3:1. The same reaction described here occurred over the pH range 7.0 to 11.0, giving the same ratio of products, except that the reaction was slower at lower pH. The addition of 1 equiv of 2,2′-bipyridine (based on copper) to the reaction did not alter the results. However, Cu(II) was found to be essential for this reaction, because a separate reaction performed without Cu(II) for 1.5 h at pH 10 resulted in recovery of starting 4-*tert*-butylresorcinol (**5**).

Reaction of with Hydrazine in DMSO-d_6. To a 5 mm NMR tube containing **1** (50 mg, 0.028 mmol) in degassed DMSO-d_6 (0.6 mL) was added hydrazine (70 μL, 2.24 mmol) via syringe. The ^1H NMR spectrum recorded 20 min after mixing showed generation of 5-*tert*-butyl-2-hydroxy-1,4-benzoquinone-1-hydrazone (**3**) and 5-*tert*-butyl-1,2,4-benzenetriol (**2**) in a ratio of 6:1. Over time, signals corresponding to compound **5** increased with a concomitant decrease of signals corresponding to compound **3**. Integration of the ^1H NMR recorded after 5 weeks indicated a mixture of **3**, **2**, and **5** in a ratio of 2:1:12. **3**: ^1H NMR (DMSO-d_6) δ 1.22 (s, 9H), 5.14 (s, 1H), 6.41 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 29.8 (3C, −), 33.9 (+), 106.2 (−), 129.0 (−), 134.3 (+), 139.8 (+), 174.7 (+), 181.8 (+); HRMS (sample prepared by evaporating the mixture of **3** and **2** in a ratio of 6:1) calcd for $C_{10}H_{14}N_2O_2$ *m*/*z* (rel intensity) 194.1055, found 194.1059 (11.0%). The same experiment run without degassing showed that conversion of **3** to **5** was about 3 times faster than that of the reaction conducted under anaerobic conditions.

Reaction of 1 with Hydrazine in CD_3CN and the Effect of Added Cu(II). To a 5 mm NMR tube containing **1** (25 mg, 0.14 mmol) in CD_3CN (0.5 mL) was added hydrazine (35 μL, 1.14 mmol). The ^1H NMR spectrum recorded 2 h after mixing showed a mixture of compounds **3**, **2**, and **5** in a ratio of 10:2:1. The signals corresponding to **5** increased with time, and after 7 days, **5** was obtained as a sole product in quantitative yield. In an identical experiment except that at the 2 h time point $Cu(ClO_4)_2 \cdot 6H_2O$ (1 mg, 0.028 mmol) was added, rapid gas (N_2) bubbling from the solution was noticed. The ^1H NMR spectrum recorded 15 min after addition of Cu(II) showed that compound **5** was a single major product along with a trace of compound **3**.

Reaction of 1 with Methylhydrazine in CD_3CN. To a 5 mm NMR tube containing a solution of **1** (9 mg, 0.05 mmol) in degassed CD_3CN (1 mL) were added *tert*-butylamine (5.2 μL, 0.1 mmol) and methylhydrazine (5.3 μL, 0.1 mmol) via syringe. After 40 min, the ^1H NMR spectrum indicated complete disappearance of **1** and the formation of 4-(methylazo)-6-*tert*-butylresorcinol (**9**) and **2** in a ratio 4:1 along with a signal due to methane (δ 0.20), as confirmed by bubbling methane into CD_3CN. No further change in the product distribution was noticed over the course of 4 days. **9**: ^1H NMR (CD_3CN) δ 1.38 (s, 9H), 3.83 (s, 3H), 6.29 (s, 1H), 7.51 (s, 1H); ^{13}C NMR (CD_3CN) δ 29.9 (3C), 34.7, 54.1, 104.8, 130.1, 131.4, 131.6, 154.4, 162.5. In another experiment showing nearly pure **9** (and CH_4) after 30 min, the CD_3CN was removed in vacuo and the spectra were re-recorded in DMSO-d_6 for better characterization. **9**: ^1H NMR (DMSO-d_6) δ 1.33 (s, 9H), 3.84 (s, 3H), 6.29 (s, 1H), 7.41 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 29.4 (3C), 33.7, 53.6, 103.7, 127.6, 128.3, 130.3, 153.2, 161.0; HRMS calcd for $C_{11}H_{16}N_2O_2$ *m*/*z* (rel intensity) 208.1212, found 208.1215 (100%).

Reaction of 1 with 1,1-Dimethylhydrazine in CD_3CN. To a 5 mm NMR tube containing a solution of **1** (9 mg, 0.05 mmol) in degassed CD_3CN (1 mL) were added *tert*-butylamine (5.2 μL, 0.05 mmol) and 1,1-dimethylhydrazine (7.6 μL, 0.1 mmol) via syringe. ^1H NMR signals due to **9**, 2-hydroxymethyl-4-methylazo-6-*tert*-butylresorcinol (**11**, tentatively identified), and **2** grew as the reaction proceeded for several hours. Compounds **9** and **2** slowly disappeared from the solution during the course of 2 days, leaving compound **11** as the major product in about 25% yield along with several unidentified compounds. Apparent ^1H NMR signals for **11**: (CD_3CN) δ 1.26 (s, 9H), 3.32 (s, 3H), 4.92 (s, 2H), 6.88 (s, 1H). In another reaction, 1,1-dimethylhydrazine (23 μL, 0.3 mmol) was added to a solution of **1** (18 mg, 0.1 mmol) in CD_3CN (0.5 mL) in a 5 mm NMR tube. Once each day for 5 days, the seal was opened briefly to admit air, to induce oxidative recycling of **2** to **1**, thereby optimizing the yield of the 1,1-dimethylhydrazine-derived products. After 5 days, the red crystals formed in the reaction tube were filtered and washed with CH_3CN, giving pure 2,2′-methylenebis(4-methylazo-6-*tert*-butylresorcinol) (**12**): ^1H NMR ($CDCl_3$) δ 1.39 (s, 18H), 3.69 (d, J = 3.18 Hz, 6H), 3.80 (s, 2H), 7.10 (s, 2H), 12.1 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 17.8 (+), 29.9 (−), 34.6 (+), 43.4 (−), 116.6 (+), 129.0 (−), 131.9 (+), 133.4 (+), 166.3 (+), 168.5 (+); HRMS calcd for $C_{23}H_{32}N_4O_4$ *m*/*z* (rel intensity) 428.2426, found 428.2408 (65%).

Anaerobic Reaction of 1 with Methylhydrazine or 1,1-Dimethylhydrazine in DMSO-d_6. To a 5 mm NMR tube containing a solution of **1** (3.6 mg, 0.02 mmol) in degassed DMSO-d_6 (0.5 mL) was added methylhydrazine (2.1 μL, 0.04 mmol). ^1H NMR signals corresponding to triol **2** and what appears to be 2-hydroxy-5-*tert*-butyl-1,4-benzoquinone 1-(methylhydrazone) (**8**) [^1H NMR (DMSO-d_6) δ 1.27 (s, 9H), 2.18 (s, 3H), 6.38 (s, 1H), 6.62 (s, 1H)] were found in a ratio of 1:1 along with a trace of **9**. Integration of **2** increased with a concomitant decrease of **8** over the course of several hours at room temperature. After 24 h, **8** disappeared completely, leaving triol **2** as the major component (80%) derived from **1**. Traces of **9** and methane (δ 0.2) were also seen initially and survived at least for 2 weeks in the solution without any sign of decomposition or change.

The same reaction performed instead with 0.04 mmol of 1,1-dimethylhydrazine exhibited in the earliest ^1H NMR spectrum recorded (5 min) the same signals as seen above, tentatively assigned to **8**, as the major component (ca. 70%) along with a trace of triol **2**. After 16 h at room temperature, ^1H NMR indicated that **8** had disappeared completely, and triol **2** was found to be the major component (ca. 60%) along with several minor unidentified products.

Interaction of BPAO with Hydrazine and Its Analogues. Solutions (0.9 mL) of hydrazine, methylhydrazine, 1,1-dimethylhydrazine, 3-phenylpropylhydrazine, or formaldehyde (1−200 μM final concentration, as appropriate) in 100 mM sodium phosphate buffer, pH 7.2, were incubated with 0.1 mL of a BPAO suspension (1.2−2.4 μM final concentration) at 30 °C, with or without maintenance of anaerobic conditions by argon bubbling. Aliquots (0.1 mL) were periodically withdrawn and diluted with 1.0 mL of benzylamine (10 mM in 50 mM sodium phosphate buffer, pH 7.2) in a 1 cm cuvette (1.5 mL). The rate of oxidation of benzylamine to benzaldehyde was measured by recording the increase in absorbance at 250 nm using a UV−vis spectrometer for 1 min and compared to the rate of benzylamine oxidation in a companion control solution of enzyme without hydrazine. In some experiments, where only up to four aliquots were required, the incubations were scaled to half-volume (0.5 mL). In some anaerobic experiments, an O_2 "scrubber" was used, consisting of the addition of 10 units of glucose oxidase, 500 units of catalase, and 15 mg of glucose per 1 mL of primary incubation mixture.

In one experiment, the enzyme (1.5 μM) was incubated with or without methylhydrazine (4 μM) in a 1 mL cuvette with or without argon bubbling (using a needle projecting through a serum cap along the side of the cuvette to the bottom, and another needle for gas escape), and a concentrated solution of phenylhydrazine·HCl (8 μL of 2 mM) in degassed water was added either after 3 min or after 100 min. The spectrum was then recorded from 300 to 600 nm, and the A_{450} was determined for each of the (+) methylhydrazine cases relative to the (−) methylhydrazine cases.

Concentrations of the enzyme were estimated from the absorbance change at 250 nm for benzaldehyde formation of the control reaction, using an activity of 0.48 units/mg of protein for the pure enzyme of molecular weight 85000[28] and $\Delta\epsilon_{250} = 12800\ M^{-1}\ cm^{-1}$ for benzaldehyde. The values obtained were consistent with the results of titration of active cofactor with phenylhydrazine and measuring the inflection at 450 nm.

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Experiments showing that the temporary inhibition of the enzyme by hydrazines is not due to small molecules present in solution involved rapid application of the incubation mixtures, at the time of maximum activity loss, to a column (1 × 7.8 cm) of PDX G.F. 25 equilibrated with 100 mM pH 7.2 sodium phosphate buffer, elution with buffer to obtain the enzyme fraction, and re-assay of the activity, all within 15 min following initial application to the column. Control runs established which central cut from the column contained the enzyme, and the activity was always reported as a percent of a control preparation treated in the identical manner.

Preparation of 3-Phenylpropylhydrazine. According to a modification of standard methods,[29] 98% anhydrous hydrazine (3.2 g, 100 mmol) was added to a solution of 3-phenylpropyl bromide (5.0 g, 25 mmol) in EtOH (60 mL). The mixture was heated at reflux overnight, allowed to cool, and then concentrated. To the residue was added aqueous NaOH (1 N, 50 mL), and the aqueous layer was extracted with CH_2Cl_2 (50 mL × 3). The organic layer was separated, dried over Na_2SO_4, and concentrated. The residue was subjected to column chromatography using MeOH−CH_2Cl_2 (2:1, v/v) as eluant to afford 3-phenylpropylhydrazine[29] as the free base. The crude product was diluted with MeOH (20 mL), 5 mL of concentrated HCl was added, the solution was stirred for 20 min, and the reaction mixture was concentrated. The residue was recrystallized with MeOH and a little ether to give 3-phenylpropylhydrazine dihydrochloride (2.5 g, 45%): ^{1}H NMR (D_2O) δ 1.94 (q, 2H), 2.68 (t, 2H), 3.09 (t, 2H), 7.25−7.36 (5H); ^{13}C NMR (D_2O) δ 25.8, 31.8, 50.7, 126.5, 128.5 (2C), 128.8 (2C), 140.8; FAB HRMS calcd for $C_9H_{15}N_2$ $(M + H)^+$ *m/z* 151.1235, found 151.1229 (93%)

Identification of Product by the Reaction of BPAO with 3-Phenylpropylhydrazine. A solution (1 mL) containing 3-phenylpropylhydrazine (50 μM) and BPAO (2.4 μM) in 80 mM pH 7.2 sodium phosphate buffer was incubated at 30 °C for 20 h. A companion incubation was conducted identically but lacking the enzyme. After the incubations, the solutions were extracted with ether (5 mL × 3). The organic layers were separated, dried over Na_2SO_4, and concentrated. GC-MS analysis of the residue showed a single peak corresponding to a molecular ion of 120 and major fragmentation peaks of 91, 78, and 65 only in the case of the reaction containing BPAO. The same characteristics were observed for authentic propylbenzene. No volatile products were present in the extract obtained from the control reaction.

Identification of Formaldehyde as a Product of Metabolism of 1,1-Dimethylhydrazine by BPAO. A solution (0.5 mL) containing 1,1-dimethylhydrazine (100 μM) and BPAO (2.4 μM) in 80 mM pH 7.2 sodium phosphate buffer was incubated at 30 °C for 18 h. Two companion incubations were conducted identically, one lacking the enzyme and the other lacking the 1,1-dimethylhydrazine. After the incubations, the solutions were quenched with an equal volume of a saturated solution of 2,4-dinitrophenylhydrazine in 2 M HCl and then extracted with 1 mL of CH_2Cl_2. TLC analysis of the extract showed the presence of a fast-moving derivative with a retention time identical to that for the authentic formaldehyde 2,4-dinitrophenylhydrazine, whereas no derivatives were found in the extracts obtained from the two control reactions.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for **3**, **9**, and **12**. This material is available free of charge via the Internet at http://www.pubs.acs.org.

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