Inactivation of Monomeric Sarcosine Oxidase by Reaction with *N*-(Cyclopropyl)glycine†

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Received June 21, 2000; Revised Manuscript Received August 28, 2000

ABSTRACT: Monomeric sarcosine oxidase (MSOX) catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine) and contains covalently bound flavin adenine dinucleotide (FAD). The present study demonstrates that *N*-(cyclopropyl)glycine (CPG) is a mechanism-based inhibitor. CPG forms a charge transfer complex with MSOX that reacts under aerobic conditions to yield a covalently modified, reduced flavin (λ_{max} = 422 nm, ϵ_{422} = 3.9 mM^{-1} cm^{-1}), accompanied by a loss of enzyme activity. The CPG-modified flavin is converted at an 8-fold slower rate to 1,5-dihydro-FAD ($EFADH_2$), which reacts rapidly with oxygen to regenerate unmodified, oxidized enzyme. As a result, CPG-modified MSOX reaches a CPG-dependent steady-state concentration under aerobic conditions and reverts back to unmodified enzyme upon removal of excess reagent. No loss of activity is observed under anaerobic conditions where $EFADH_2$ is formed in a reaction that goes to completion at low CPG concentrations. Aerobic denaturation of CPG-modified enzyme yields unmodified, oxidized flavin at a rate similar to the anaerobic denaturation reaction, which yields 1,5-dihydro-FAD. The CPG-modified flavin can be reduced with borohydride, a reaction that blocks conversion to unmodified flavin upon removal of excess CPG or enzyme denaturation. The possible chemical mechanism of inactivation and structure of the CPG-modified flavin are discussed.

Monomeric sarcosine oxidase (MSOX)[1] is an inducible bacterial flavoenzyme that is important in the catabolism of sarcosine (*N*-methylglycine), a common soil metabolite that can act as sole source of carbon and energy for many microorganisms (*1*). MSOX is a member of a recently recognized family of enzymes that contain covalently bound flavin and catalyze similar oxidation reactions with amine substrates. Other family members include *N*-methyltryptophan oxidase, pipecolate oxidase, and heterotetrameric sarcosine oxidase (*2*−*4*). The crystal structures of MSOX and its complexes with various substrate analogues have recently been determined (*5*, *6*). MSOX is a two-domain protein and contains FAD [8α-(*S*-cysteinyl)-FAD] covalently linked to a cysteine residue (Cys315) (*2*). The NH_2-terminal flavin domain binds the ADP portion of FAD. The "catalytic" domain contains the covalent flavin attachment site. The flavin ring and the active site are located at the domain interface.

Flavin-containing amine oxidoreductases are generally considered to share a common overall mechanism involving amine oxidation to an imine, accompanied by two-electron reduction of the flavin coenzyme. The detailed mechanism of amine oxidation has received considerable attention and is at present quite controversial. Studies with mechanism-based inactivators of monoamine oxidase have led to the proposal that amine oxidation occurs via one of two variants of a single electron transfer (SET) mechanism (*7*, *8*). The two variants both involve initial one-electron transfer from substrate amino group to enzyme flavin but differ with respect to the mechanism of the second oxidation step (H• versus H^+ plus $1e^-$) (Scheme 1). A hydride transfer mechanism has recently been proposed for D-amino acid oxidase (*9*). Amine oxidation via a polar mechanism, involving formation of a covalent flavin−substrate intermediate (4a-flavin adduct), has been observed in flavin model studies (*10*, *11*) (Scheme 1).

The MSOX active-site structure and predicted substrate binding mode are consistent with each of the four mechanisms proposed for amine oxidation. No evidence for a spectrally detectable intermediate was obtained in reductive half-reaction studies with MSOX (*12*). This result does not, however, preclude a polar or SET mechanism since the postulated intermediate might be formed in a rate-determining step. Thiols are known to form 4a-adducts (*13*), analogous to the 4a-flavin−substrate adduct in the polar mechanism. Unlike the latter, a 4a-adduct formed with MSOX and a monothiol might be stable since it could not undergo further reaction to yield unsubstituted, two-electron-reduced flavin. Adduct formation with MSOX and thioglycolate ($HSCH_2CO_2^-$) is not observed, but the compound binds to the active site and acts as a one-electron donor, converting oxidized MSOX to the anionic flavin radical (*12*).

† This work was supported in part by Grants GM 31704 (M.S.J.) and GM 57870 (F.A.D.) from the National Institutes of Health.

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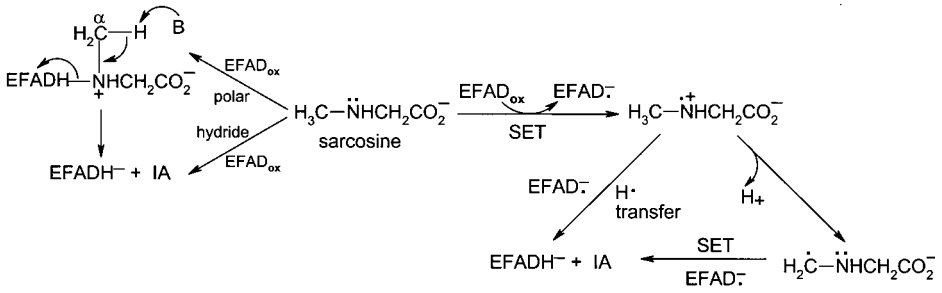
[1] Abbreviations: MSOX, monomeric sarcosine oxidase; CPG, *N*-(cyclopropyl)glycine; FAD, flavin adenine dinucleotide; SET, single electron transfer.

10.1021/bi001421w CCC: $19.00
Published on Web 10/26/2000

Scheme 1: Possible Mechanisms for Sarcosine Oxidation by MSOX[a]

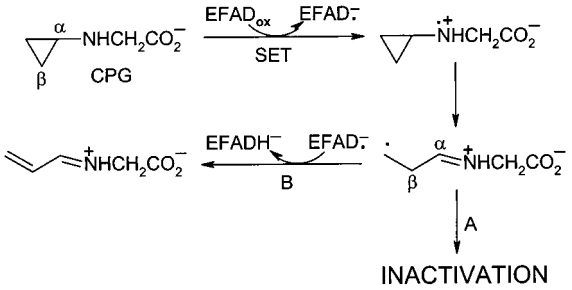


[a] IA = $CH_2{=}NH^+CH_2CO_2^-$.

Since the hydride mechanism involves nucleophilic addition of $H:^-$ at flavin N(5), this position of the MSOX flavin might react with other nucleophiles. Although nucleophilic addition of sulfite at flavin N(5) is a reaction characteristically observed with flavoprotein oxidases, studies with MSOX and other members of the MSOX family show that there is a significant kinetic barrier against sulfite addition (*6*).

The cyclopropyl group has proved useful in the study of various enzyme reactions (*14*). *N*-(Cyclopropyl)glycine (CPG) is unlikely to act as a substrate for MSOX if catalysis proceeds via a hydride mechanism because cyclopropyl derivatives are extremely unreactive in reactions involving conversion of a tetrahedral carbon to a trigonal carbon (*15*). On the other hand, reaction with CPG might facilitate detection of a 4a-flavin adduct intermediate since the cyclopropyl substituent should not interfere with reversible adduct formation (*11*) but would inhibit loss of the α-proton and therefore block further reaction via the normal polar mechanism. Cyclopropyl derivatives can undergo radical rearrangement reactions, a feature that has prompted their use as probes for detecting radical intermediates. The amine cation radical formed upon one-electron oxidation of CPG is expected to undergo rapid cyclopropyl ring opening ($k > 5 \times 10^8\ s^{-1}$) (*16*), forming a potentially reactive species that might covalently modify MSOX and cause enzyme inactivation (Scheme 2, path A). Cyclopropylamine derivatives have

Scheme 2: CPG Oxidation by a Single Electron Transfer Mechanism



previously been identified as suicide substrates for monoamine oxidase and cytochrome P-450 (*7*, *17*).

In this paper we show that CPG is a mechanism-based inhibitor of MSOX from *Bacillus* sp. B-0618 and inactivates the enzyme by covalently modifying the flavin. The kinetic mechanism of the inactivation process has been determined.

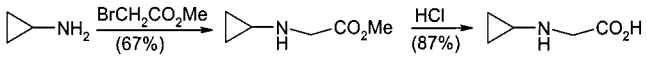
EXPERIMENTAL PROCEDURES

Materials. Horseradish peroxidase and *o*-dianisidine were purchased from Sigma. Sarcosine, cyclopropylamine, and methyl bromoacetate were obtained from Aldrich. Guanidine hydrochloride was from Fisher. Microcon YM-10 microconcentrators were obtained from Amicon.

Purification and Assay of Recombinant MSOX. *Escherichia coli* strain DH1/pMAW was grown at 37 °C in LB medium containing carbenicillin as previously described (*2*). Enzyme purification and routine protein and activity assays were performed as previously described (*2*). The horseradish peroxidase-coupled assay described by Wagner and Jorns (*12*) was used to investigate the effect of CPG treatment on MSOX activity.

Spectroscopy. Absorption spectra were recorded on a Perkin-Elmer Lambda 2S spectrometer. All spectra are corrected for dilution. The previously determined extinction coefficients for MSOX at 454 nm ($\epsilon_{454} = 12\ 200\ M^{-1}\ cm^{-1}$) and free FAD in 3 M guanidine hydrochloride ($\epsilon_{450} = 11\ 900\ M^{-1}\ cm^{-1}$) (*2*) were used to calculate values for the CPG-modified flavin bound to native or denatured MSOX, respectively. Extinction coefficients were determined for CPG-modified enzyme prepared by aerobic reaction of MSOX with 200 mM CPG; observed values were not corrected for the estimated 94% conversion to CPG-modified enzyme under these conditions. Anaerobic experiments were conducted as previously described (*6*).

CPG Synthesis and Storage. CPG was synthesized via a two-step procedure. In a 100 mL single-neck round-bottom



flask, equipped with a magnetic stir bar, rubber septum, and an argon balloon, was placed 1.48 g (26.0 mmol) of cyclopropylamine in dry ether (5 mL). The mixture was cooled to 5−10 °C and a solution of 1.70 g (11.1 mmol) of methyl bromoacetate in ether (5 mL) was added dropwise. After stirring at 5−10 °C for 1.5 h, the solution was warmed to room temperature and stirred for 8 h. The reaction mixture was filtered to remove the cyclopropylamine hydrobromide, the filtrate was concentrated, and the residue was distilled at 50 °C (1.5 mm Hg) in a Kugelrohr apparatus to give 0.96 g (67%) of methyl *N*-(cyclopropyl)glycinate as a colorless liquid; IR (thin film) 3343 and 1745 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.34−0.44 (m, 4H), 2.15 (br s, 1H), 2.20−2.23 (m, 1H), 3.46 (s, 2H), and 3.73 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 172.8, 50.3, 50.1, 29.5, and 6.1.

In a 250 mL single-neck round-bottom flask, equipped with a magnetic stir bar and a reflux condenser, was placed 0.80 g (6.2 mmol) of *N*-(cyclopropyl)glycinate, and 3 N HCl (70 mL) was added. The reaction mixture was refluxed for

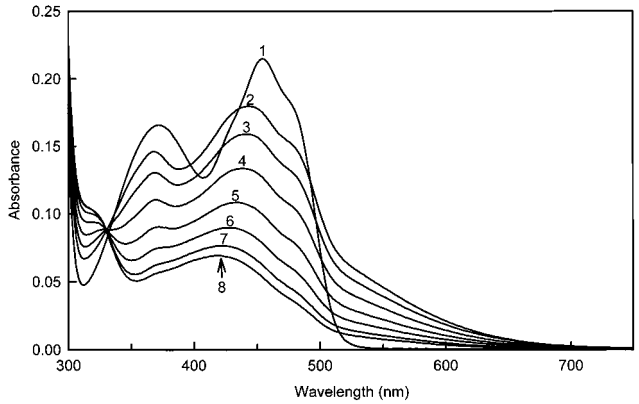


FIGURE 1: Aerobic modification of MSOX with CPG. Curve 1 is the spectrum of unmodified MSOX in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C. Curves 2−8 were recorded 0.85, 3.35, 7.0, 12.5, 20.0, 32.0, and 65.0 min, respectively, after adding 200 mM CPG.

2 h, cooled to room temperature, concentrated, and passed through a Dowex 50 × 8-100 ion-exchange resin, eluting with 1.5 M NH_4OH (120 mL). The aqueous solution was concentrated on a rotary evaporator with a bath temperature of approximately 60 °C to afford 0.61 g (87%) of CPG as a white solid: mp 230 °C (decomp); IR (KBr) 3443−2360 and 1582 cm^{-1}; ^1H NMR (D_2O) δ 3.66 (s, 2H), 2.71−2.72 (m, 1H), and 0.82−0.84 (m, 4H); ^{13}C NMR (DMSO-d_6 + D_2O) δ 169.7, 51.0, 30.7, and 4.0. Anal. Calcd. for C_5H_8NO: C, 52.16; H, 7.88; N, 12.17. Found: C, 52.12; H, 8.09; N, 12.25.

Stock solutions of CPG were prepared in 50 mM potassium phosphate buffer, pH 8.0, stored at 4 °C, and used within 2 weeks. Control reactions were always conducted with CPG from the same stock solution.

Data Analysis. Data were fit to eqs 1−3 by use of the curve-fit function in SigmaPlot (Jandel Corp.). Equation 1

$$Y = Ae^{-kt} + B \quad (1)$$

was used to fit first-order reaction kinetics. Y is the observed absorbance at the selected wavelength and time = t, A is the maximal absorbance change, and B is the final absorbance at the selected wavelength. Equations 2 and 3 are described in the Results section.

Miscellaneous. Filtration of reaction mixtures to remove protein was performed on Microcon YM-10 microconcentrators. Guanidine hydrochloride solutions were prepared in 50 mM potassium phosphate buffer, pH 8.0.

RESULTS

Aerobic Reaction of MSOX with 200 mM CPG. CPG binds to MSOX and forms a charge-transfer complex, as judged by the appearance of an intense new absorption band in the long-wavelength region (Figure 1, curve 2). Under aerobic conditions, the charge-transfer complex undergoes an isosbestic conversion to a modified reduced flavin species (λ_{max} = 422 nm) (Figure 1, curve 8). The CPG-modified flavin is air-stable and exhibits different spectral properties as compared with 1,5-dihydroflavin, an oxygen-sensitive species that is formed as a normal intermediate during turnover of MSOX with sarcosine (see Figure 6, inset).

Table 1: Observed Rates of Flavin Modification and Enzyme Inactivation during Aerobic Reaction of MSOX with CPG[a]

	k_{obs} (min^{-1})	
[CPG] (mM)	modification	activity loss
50	0.051 ± 0.002	0.055 ± 0.003
100	0.064 ± 0.002	0.066 ± 0.003
150	0.077 ± 0.002	0.077 ± 0.007

[a] Reactions were conducted in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C.

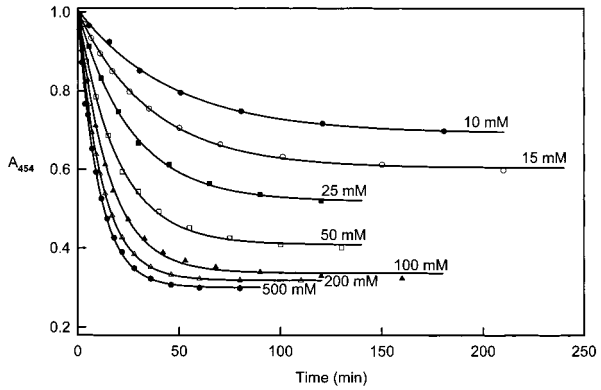


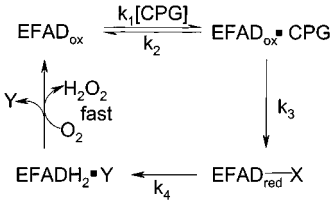
FIGURE 2: Effect of CPG concentration on the rate and extent of aerobic modification of MSOX. Reactions were conducted in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C, and the indicated CPG concentration. The solid lines show the fit of absorbance changes at 454 nm to a single-exponential expression ($y = Ae^{-kt} + B$). The data at different CPG concentrations were normalized to the same enzyme concentration by setting $A + B = 1$.

Effect of CPG on MSOX Activity. The aerobic reaction of MSOX with CPG is accompanied by a loss of enzyme activity. The loss of activity and flavin modification exhibit apparent first-order kinetics. Both processes occur at the same rate (Table 1). The results indicate that enzyme inactivation is probably due to flavin modification.

Effect of CPG Concentration on the Rate and Extent of Flavin Modification. Both the rate and extent of flavin modification observed upon aerobic reaction of MSOX with CPG are dependent on the inhibitor concentration. As shown in Figure 2, reactions at less than about 200 mM CPG appear to stop before the modification is complete. If MSOX underwent multiple turnovers prior to a modification event, CPG might be consumed before the modification reaction was complete. To test this hypothesis, MSOX was allowed to react with 50 mM CPG until no further spectral changes were observed (cycle 1). The solution was microfiltered to remove MSOX. The filtrate was then mixed with a fresh aliquot of MSOX (cycle 2). The results do not support the hypothesis since the same rate of flavin modification was observed in cycle 1 (0.0500 ± 0.0006 min^{-1}) and cycle 2 (0.0507 ± 0.0003 min^{-1}).

The kinetic mechanism shown in Scheme 3 provides an alternate explanation for the effect of CPG on the rate and extent of flavin modification. In this mechanism, the aerobic reaction of MSOX with CPG involves initial formation of a noncovalent charge-transfer complex ($EFAD_{ox}$·CPG). This complex reacts to form a covalently modified reduced flavin ($EFAD_{red}$-X) in a CPG-dependent but oxygen-independent reaction. The covalently modified reduced flavin can, however, rearrange to form normal 1,5-dihydro-FAD plus Y that is noncovalently bound to MSOX ($EFADH_2$·Y). This

Scheme 3: Kinetic Mechanism for MSOX Modification by CPG[a]



[a] See text for details.

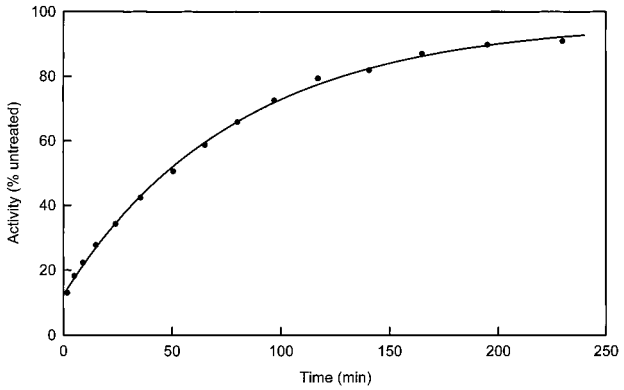


FIGURE 3: Recovery of activity upon dilution of CPG-modified MSOX. MSOX was inactivated by reaction with 200 mM CPG in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C. The modified enzyme was diluted 2000-fold into the same buffer without CPG and assayed after incubation for various periods of time at 25 °C. The solid line is a fit of activity data to a single-exponential expression.

species reacts rapidly with oxygen to regenerate unmodified, oxidized MSOX (EFAD$_{ox}$). As a result, CPG-modified MSOX reaches a steady-state concentration that depends on the inhibitor concentration.

Scheme 3 generates several testable predictions. First, a net conversion of CPG-modified MSOX to unmodified enzyme should occur when the CPG concentration is decreased, either by a large dilution or by dialysis. In one experiment, CPG-modified enzyme was formed by reaction with 200 mM CPG. The sample was then diluted 1000-fold and the conversion to unmodified MSOX was monitored by measuring enzyme activity. Figure 3 shows that dilution from 200 to 0.2 mM CPG results in the recovery of MSOX activity in an apparent first-order reaction [k_{obs} (k_4) = 0.0126 ± 0.0005 min^{-1}]. In another experiment, enzyme modified with 200 mM CPG was dialyzed to remove excess inhibitor. The dialyzed sample exhibited spectral and catalytic properties similar to those of untreated MSOX. The results provide further evidence that enzyme inactivation is due to flavin modification.

According to Scheme 3, the observed rate of formation of CPG-modified enzyme under aerobic conditions should exhibit the CPG concentration dependence shown in eq 2,

$$k_{obs} = \frac{k_3[\mathrm{CPG}]}{K_i + [\mathrm{CPG}]} + k_4 \qquad (2)$$

where $K_i = (k_2 + k_3)/k_1$. Figure 4 shows a plot of $k_{obs} - k_4$ versus the CPG concentration, where k_4 was set equal to the rate of activity return upon dilution of CPG-modified enzyme (k_4 = 0.0126 min^{-1}). The data gave a good fit to eq 2 (K_i =

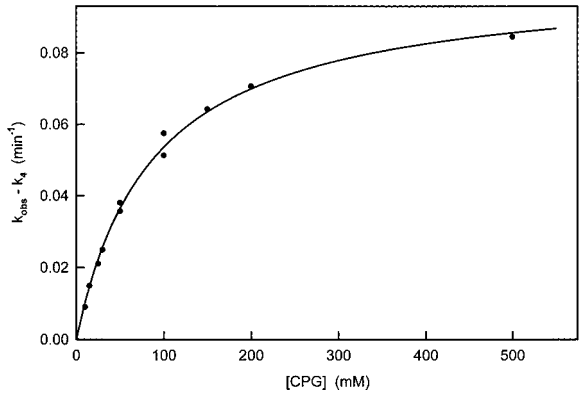


FIGURE 4: Effect of CPG concentration on the observed rate of aerobic modification of MSOX. The solid line is the fit of the data to eq 2, using the independently determined value obtained for k_4 (0.0126 min^{-1}) as described in the text. This figure includes values shown in Table 1 plus additional data points.

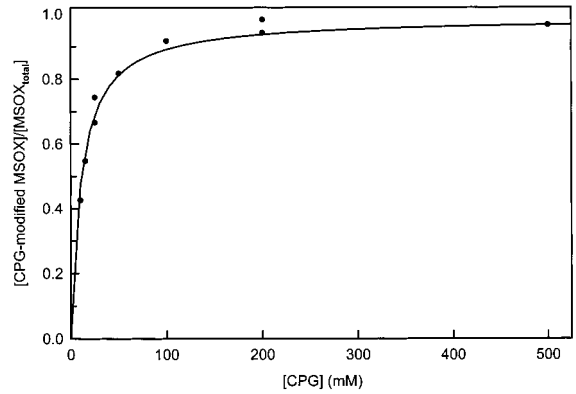


FIGURE 5: Effect of CPG concentration on the fraction of CPG-modified MSOX at steady state. The solid line is the fit of the data to eq 3, using values determined for k_3 (0.101 min^{-1}), K_i (87 mM), and k_4 (0.0126 min^{-1}), as described in the text. Values for the fraction of CPG-modified MSOX were estimated from the observed decrease in A_{454} and normalized to the calculated value at 500 mM CPG.

87 ± 5 mM; k_3 = 0.101 ± 0.003 min^{-1}). Similar results were obtained when the data were fit to eq 2 without assuming a value for k_4 (K_i = 78 ± 8 mM; k_3 = 0.101 ± 0.001 min^{-1}; k_4 = 0.010 ± 0.002). According to Scheme 3, the yield of CPG-modified enzyme at steady state under aerobic conditions should exhibit the CPG concentration dependence shown in eq 3

$$\frac{[\mathrm{EFAD_{red}\text{-}X}]}{[\mathrm{E_{total}}]} = \frac{k_3[\mathrm{CPG}]}{k_4K_i + [\mathrm{CPG}](k_3 + k_4)} \qquad (3)$$

As shown in Figure 5, a good fit to eq 3 was obtained with values determined for K_i, k_3, and k_4, as described above.

Anaerobic Reaction of MSOX with CPG. According to Scheme 3, CPG-modified enzyme should not accumulate under anaerobic conditions because all of the enzyme will be "trapped" as EFADH$_2$ (or EFADH$_2$·Y). Scheme 3 also predicts that complete conversion to EFADH$_2$ should be observed even at low CPG concentrations. The spectral course of the anaerobic reaction of MSOX with 5 mM CPG is shown in Figure 6. A nearly isosbestic conversion to EFADH$_2$ is observed. The rate of EFADH$_2$ formation (k_{obs} = 0.009 ± 0.001 min^{-1}) is similar to the rate determined for k_4 under aerobic conditions (0.0126 min^{-1}). The absorp-

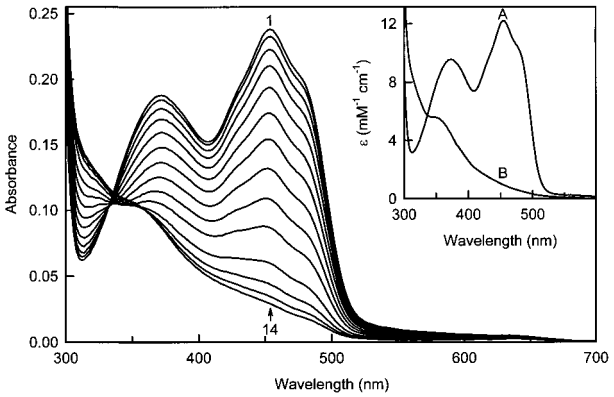


FIGURE 6: Anaerobic reaction of MSOX with CPG or sarcosine. Curves 1−14 were recorded 2, 5, 10, 17, 27, 40, 55, 75, 100, 130, 196, 243, 302, and 363 min, respectively, after addition of 5 mM CPG to MSOX in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C. Inset: Curves A and B were recorded before and immediately after MSOX was mixed with 80 mM sarcosine under the same conditions.

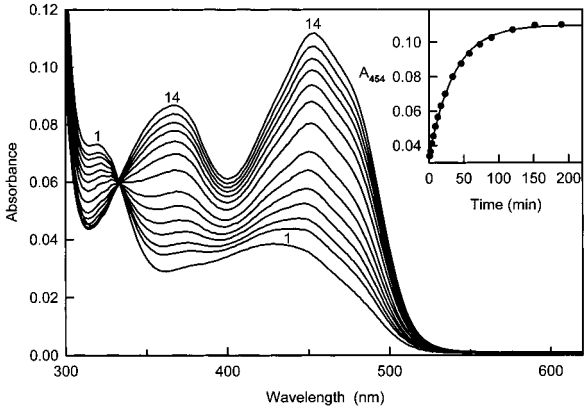


FIGURE 7: Aerobic denaturation of CPG-modified MSOX. MSOX was reacted under aerobic conditions with 200 mM CPG in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C. The CPG-modified enzyme was then diluted 1:1 with aerobic 6 M guanidine hydrochloride. Curves 1−14 were recorded 0.85, 4.0, 6.0, 9.0, 12.5, 17.1, 23.0, 34.0, 46.1, 58.0, 73.2, 90.0, 120.0, and 190.0 min, respectively, after mixing. The inset shows a plot of the absorbance increase at 454 nm. The solid line is a fit of the data to a single-exponential expression.

tion spectrum of the CPG-reduced enzyme is very similar to that observed upon anaerobic reduction with sarcosine (Figure 6, inset). Immediate flavin reoxidation is observed upon exposure of the CPG-reduced enzyme to air. No loss of MSOX activity is observed, even after prolonged incubation under anaerobic conditions, when the enzyme is assayed immediately after aeration.

Denaturation of CPG-Modified MSOX under Aerobic or Anaerobic Conditions. CPG-modified MSOX was prepared by aerobic reaction with 200 mM CPG and then denatured by a 1:1 dilution with aerobic 6.0 M guanidine hydrochloride. Aerobic denaturation results in an isosbestic conversion to unmodified, oxidized flavin in an apparent first-order reaction ($k_{obs(aerobic,denatured)} = 0.028 \pm 0.007$ min^{-1}) (Figure 7), analogous to the reaction observed upon 1000-fold dilution of intact CPG-modified enzyme ($k_4 = 0.0126$ min^{-1}) except for a 2-fold difference in rate. According to Scheme 3, these reactions should proceed via a 1,5-dihydro-FAD intermediate. To test this hypothesis, a concentrated sample of CPG-modified enzyme was prepared by reaction with 200 mM

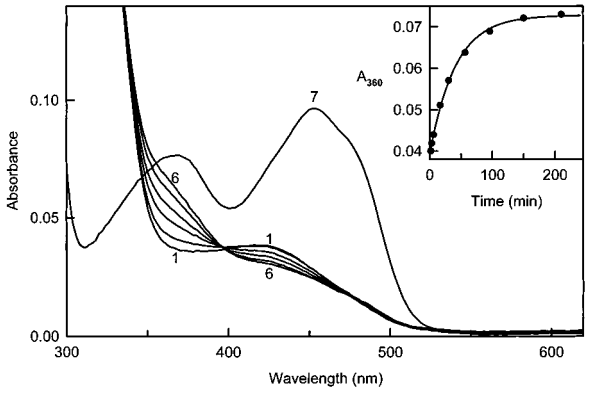


FIGURE 8: Anaerobic denaturation of CPG-modified MSOX. MSOX (424 μM) was reacted under aerobic conditions with 200 mM CPG in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C. The CPG-modified enzyme was diluted 1:40 into anaerobic 3 M guanidine hydrochloride. Curves 1−6 were recorded 1.7, 6.0, 16.5, 30.1, 56.5, and 96.0 min, respectively, after mixing. Curve 7 was recorded immediately after aeration. The inset shows a plot of the absorbance increase at 360 nm under anaerobic conditions. The solid line is a fit of the data to a single-exponential expression.

CPG under aerobic conditions. A small aliquot (15 μL) was transferred to the sidearm of a special cuvette (*6*) that contained 585 μL of 3 M guanidine hydrochloride in the main compartment. The cuvette was made anaerobic. CPG-modified MSOX was then diluted 40-fold into the denaturant. Anaerobic denaturation results in the isosbestic conversion to a species with spectral properties similar to those of 1,5-dihydro-FAD (Figure 8). The reaction exhibited apparent first-order kinetics (Figure 8, inset) and occurred at a rate ($k_{obs(anaerobic,denatured)} = 0.025 \pm 0.002$ min^{-1}) similar to that observed upon aerobic denaturation ($k_{obs(aerobic,denatured)} = 0.028 \pm 0.007$ min^{-1}). Exposure of the anaerobic sample to air resulted in the immediate conversion to unmodified oxidized flavin (Figure 8, curve 7).

Reaction of CPG-Modified MSOX with Sodium Borohydride. The flavin chromophore in CPG-modified MSOX can be further reduced by reaction with sodium borohydride, as judged by the loss of the 422 nm absorption band (Figure 9, curve 3). The borohydride-reduced CPG-modified flavin does not revert to unmodified oxidized flavin upon dialysis (curve 4, $\lambda_{max} = 324$ nm), unlike that observed for untreated CPG-modified enzyme. Denaturation of borohydride-reduced CPG-modified flavin with 3 M guanidine hydrochloride resulted in an immediate shift in the maximum from 324 to 320 nm, accompanied by a 6% increase in absorbance (data not shown). A slow secondary spectral change resulted in the loss of the 320 nm peak, accompanied by small increase in absorbance at 454 nm in a reaction that was complete in 7 h. The final spectrum is shown in Figure 9, curve 5. No recovery of unmodified oxidized flavin was observed, unlike that observed upon denaturation of untreated CPG-modified enzyme. In a control reaction, the flavin in oxidized MSOX was converted to 1,5-dihydro-FAD by mixing with excess sarcosine (20 mM) in aerobic buffer. No spectral change was observed upon addition of 60 mM sodium borohydride. Unmodified oxidized enzyme was recovered after an overnight incubation at room temperature.

DISCUSSION

CPG appears to act as a mechanism-based inhibitor of MSOX (Scheme 3). The compound binds to the active site

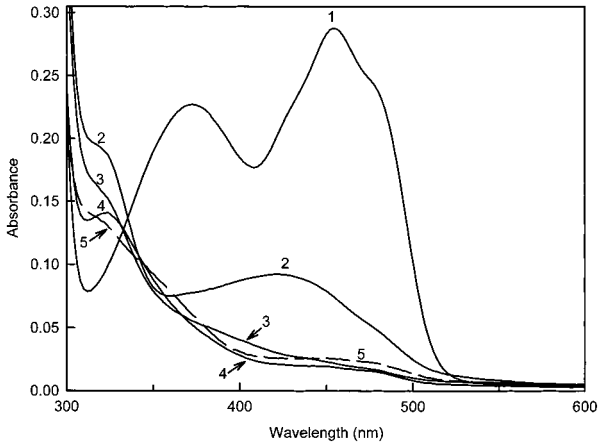
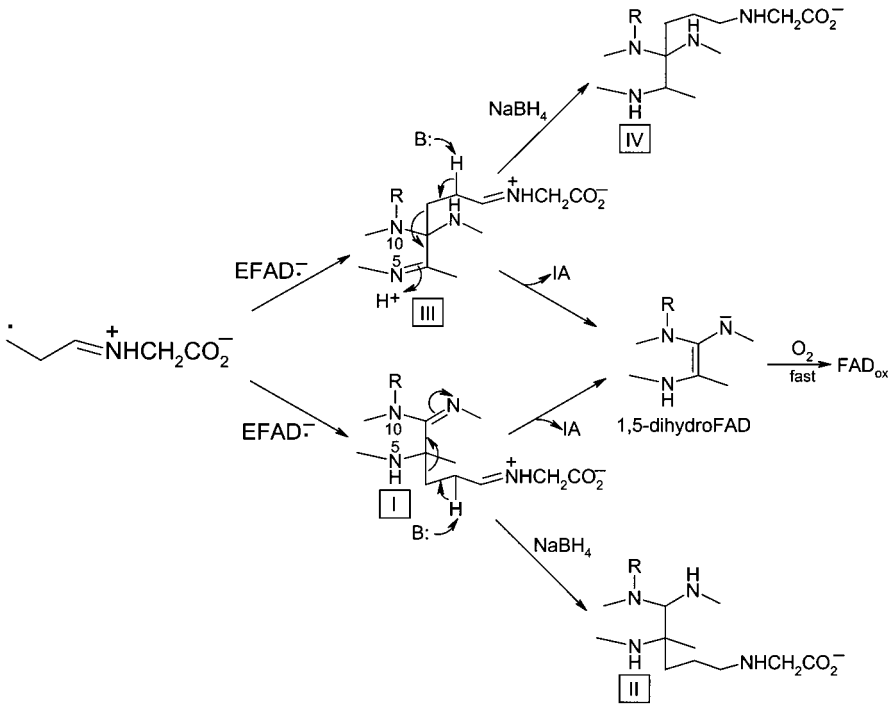


FIGURE 9: Effect of sodium borohydride reduction on the stability of CPG-modified MSOX. Curve 1, untreated MSOX in 50 mM potassium phosphate buffer, pH 8.0 at 25 °C; curve 2, after reaction with 200 mM CPG; curve 3, after reaction of CPG-modified MSOX with 60 mM sodium borohydride; curve 4, after dialysis of borohydride-treated CPG-modified MSOX; curve 5 (dashed line), 420 min after denaturation of borohydride-treated CPG-modified MSOX with 3 M guanidine hydrochloride.

and forms a noncovalent, charge-transfer complex with FAD as the charge transfer acceptor. Under aerobic conditions, this complex reacts to form a covalently modified, reduced flavin, accompanied by a loss of enzyme activity ($k_3 = 0.101$ min^{-1}). Although the covalent modification step is irreversible, the CPG-modified flavin can react in a relatively slow reaction ($k_4 = 0.0126$ min^{-1}) to yield 1,5-dihydro-FAD ($EFADH_2$). $EFADH_2$ reacts rapidly with oxygen to regenerate unmodified, oxidized MSOX, which can now react with a second molecule of CPG. As a result, CPG-modified MSOX reaches a CPG-dependent steady-state concentration under aerobic conditions and reverts back to unmodified enzyme upon removal of excess reagent via dialysis or dilution. Under anaerobic conditions MSOX is converted to $EFADH_2$ with no loss of activity, even after prolonged incubation. Unlike aerobic modification, the anaerobic reaction goes to completion even at low CPG concentrations, consistent with Scheme 3. It is possible, however, that $EFADH_2$ formation under anaerobic conditions may occur partly via a pathway that does not involve the intermediate formation of CPG-modified flavin (e.g., Scheme 2, path B). Conversion to unmodified, oxidized flavin is observed upon aerobic denaturation of CPG-modified enzyme in a reaction that is about 2-fold faster ($k_{obs(aerobic,denatured)} = 0.028$ min^{-1}) than the corresponding reaction observed by removing excess CPG under nondenaturing conditions ($k_4 = 0.0126$ min^{-1}). The 2-fold difference in reaction rates suggests that the CPG-modified flavin is somewhat stabilized by the active-site environment. Anaerobic denaturation of CPG-modified enzyme results in the formation of 1,5-dihydro-FAD at a rate ($k_{obs(anaerobic,denatured)} = 0.025$ min^{-1}) similar to that observed for the aerobic reoxidation reaction. The results support the proposed formation of $EFADH_2$ as an intermediate in the recycling of CPG-modified MSOX, as shown in Scheme 3. The CPG-modified flavin can be reduced with borohydride, a reaction that prevents conversion back to unmodified flavin upon removal of excess CPG or enzyme denaturation.

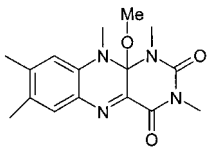
The observed inactivation of MSOX is consistent with CPG oxidation via a SET mechanism. One-electron oxidation of CPG would generate a flavin radical plus an amine cation radical that can to undergo rapid cyclopropyl ring opening to yield a ring-cleaved radical (Scheme 2). The inhibitor radical might inactivate the enzyme by reacting with the flavin radical to yield a covalently modified reduced flavin. Formation of a C(4a)-alkylated 4a,5-dihydroflavin (Scheme 4, compound I) would be in accord with results obtained in photochemical studies with free flavin and a cyclopropylamine derivative where a similar alkylated reduced flavin

Scheme 4: Possible Structures for the CPG-Modified Flavin and Its Reaction Products[a]



[a] IA = CH_2═CHCH═$NH^+CH_2CO_2^-$.

Table 2: Spectral Properties of the CPG-Modified Flavin and a Possible Model Compound

compound	spectral properties[a] λ_{max} (nm)	ϵ (mM^{-1} cm^{-1})
Model Compound		
	423	4.7
	312	7.8
CPG-Modified Flavin		
Denatured MSOX	428	4.1
	304	10.6
Native MSOX	422	3.9
	317 (s)	5.9

[a] Spectral properties of the model compound were reported in methanol (*22*). Data for native or denatured CPG-modified MSOX were obtained in 50 mM potassium phosphate, pH 8.0, containing 0 or 3 M guanidine hydrochloride, respectively.

was formed via a SET mechanism involving the flavin triplet (*18*). Compound I might undergo a base-catalyzed retro-Michael reaction to generate 1,5-dihydro-FAD, which can react with oxygen to yield unmodified, oxidized FAD, as observed with the CPG-modified flavin in MSOX. Borohydride is expected to reduce the imine linkages and convert compound I to compound II. Loss of the imine linkages should block the retro-Michael rearrangement reaction, consistent with the effect of borohydride on the stability of the CPG-modified flavin. However, the spectral properties of the CPG-modified flavin do not appear to be in accord with those reported for known derivatives similar to compound I; these derivatives exhibit absorption maxima at considerably shorter wavelengths (λ_{max} = 365−385 nm) (*19*) as compared with the CPG-modified flavin (Table 2). Addition of the inhibitor at flavin N(5) is even less likely than C(4a) addition since N(5)-alkylated 1,5-dihydroflavin derivatives absorb at even shorter wavelengths (λ_{max} = 325−350 nm) (*19*). Compound III in Scheme 4 might be formed by addition of the inhibitor radical at flavin C(10a), a position that also carries spin density (*20*). Derivatives similar to compound III exhibit spectral properties similar to those of the CPG-modified flavin (Table 2). Compound III can rearrange to yield 1,5-dihydro-FAD or react with borohydride, as described for compound I, except that elimination of an N(5)-flavin anion (high pK_a conjugate acid) would be less favorable than the elimination of a C(4a) flavin anion (low pK_a conjugate acid). Inactivation of monoamine oxidase with 1-phenylcyclopropylamine has been shown to involve cyclopropyl ring opening and formation of a modified reduced flavin (*21*), similar to that proposed for the MSOX reaction with CPG. However, unlike the MSOX reaction, a stable modified flavin was formed with monoamine oxidase, accompanied by irreversible inactivation; attachment of the inhibitor at the N(5) position was suggested on the basis of the spectral properties of the isolated flavin peptide.

The modified reduced flavins discussed above might be formed via a Michael addition reaction of 1,5-dihydro-FAD with the electrophilic α,β-unsaturated imine formed by two-electron oxidation of CPG, accompanied by ring opening (e.g., Scheme 2, path B). This possibility is considered unlikely since the same electrophilic α,β-unsaturated imine should be formed during turnover of MSOX with *N*-(allyl)-glycine (CH_2═$CHCH_2NHCH_2CO_2^-$), a reaction that is not accompanied by a CPG-like modification of the MSOX flavin.[2] MSOX inactivation by a mechanism involving rebinding of an electrophilic species from solution can be ruled out since the aerobic reaction with CPG is unaffected by glutathione (10 mM), a reagent that does not bind to MSOX but would scavenge any α,β-unsaturated imine or aldehyde released into solution.[3] The chemical mechanism of MSOX inactivation by CPG is under investigation, as are crystallographic studies to determine the structure of the borohydride-reduced CPG-modified flavin. The latter has been crystallized and a data set at 1.85 Å resolution is currently being processed.[4]

[2] G. Zhao, J. Qu, F. A. Davis, and M. S. Jorns, unpublished results.
[3] G. Zhao and M. S. Jorns, unpublished results.
[4] Z.Chen, G. Zhao, M. S. Jorns, and F. S. Mathews, unpublished results.

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BI001421W