15444 *Biochemistry* **2005,** *44,* 15444−15450

Structure of the Sodium Borohydride-Reduced *N*-(Cyclopropyl)glycine Adduct of the Flavoenzyme Monomeric Sarcosine Oxidase[†,‡]

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Received August 2, 2005; Revised Manuscript Received September 8, 2005

ABSTRACT: Monomeric sarcosine oxidase (MSOX) is a flavoprotein that contains covalently bound FAD [8a-(*S*-cysteinyl)FAD] and catalyzes the oxidation of sarcosine (*N*-methylglycine) and other secondary amino acids, such as L-proline. Our previous studies showed that *N*-(cyclopropyl)glycine (CPG) acts as a mechanism-based inactivator of MSOX [Zhao, G., et al. (2000) *Biochemistry 39*, 14341−14347]. The reaction results in the formation of a modified reduced flavin that can be further reduced and stabilized by treatment with sodium borohydride. The borohydride-reduced CPG-modified enzyme exhibits a mass increase of 63 ± 2 Da as compared with native MSOX. The crystal structure of the modified enzyme, solved at 1.85 Å resolution, shows that FAD is the only site of modification. The modified FAD contains a fused five-membered ring, linking the C(4a) and N(5) atoms of the flavin ring, with an additional oxygen atom bound to the carbon atom attached to N(5) and a tetrahedral carbon atom at flavin C(4) with a hydroxyl group attached to C(4). On the basis of the crystal structure of the borohydride-stabilized adduct, we conclude that the labile CPG-modified flavin is a 4a,5-dihydroflavin derivative with a substituent derived from the cleavage of the cyclopropyl ring in CPG. The results are consistent with CPG-mediated inactivation in a reaction initiated by single electron transfer from the amine function in CPG to FAD in MSOX, followed by collapse of the radical pair to yield a covalently modified 4a,5-dihydroflavin.

Monomeric sarcosine oxidase (MSOX)[1] is a flavoprotein that contains covalently bound FAD [8a-(*S*-cysteinyl)FAD] (*2*). The enzyme catalyzes the oxidation of sarcosine (*N*-methylglycine) and other secondary amino acids, such as L-proline. Sarcosine is a common soil metabolite and induces MSOX expression in various bacteria grown with sarcosine as the sole source of carbon and energy (*3*). MSOX is a member of a family of enzymes that contain covalently bound flavin and catalyze similar oxidation reactions with different amine substrates (*4*−*7*). The crystal structure of free MSOX from *Bacillus sp. B-0618*, one of its mutants and complexes of the enzyme with various inhibitors have been determined (*8*−*10*). MSOX is a two-domain, 46 kDa protein with an overall topology similar to D-amino acid oxidase.

Despite considerable attention, important questions regarding the mechanism of flavin-dependent amine oxidation reactions remain unresolved. Postulated mechanisms exhibit different geometric constraints with respect to the orientation of flavin, substrate, and a putative active site base. The mechanisms also differ with respect to the acceptor of hydrogen abstracted from the carbon atom in the carbon−nitrogen bond undergoing oxidation. We initiated studies on the reaction of MSOX with *N*-(cyclopropyl)glycine (CPG) because different outcomes are predicted, depending on the mechanism of substrate oxidation (Scheme 1). In the hydride mechanism, substrate hydrogen is transferred to the N(5) position of the flavin ring. This mechanism is not feasible with CPG as substrate because it would involve a highly unfavorable change in the hybridization of the cyclopropyl ring carbon from tetrahedral to trigonal (*11*). Polar mechanisms are initiated by nucleophilic addition of substrate amino group at flavin C(4a) in a reversible reaction that yields a covalent flavin-substrate adduct. The 4a-adduct is converted to oxidized amine and 1,5-dihydroflavin in a second step that requires abstraction of substrate hydrogen by an active site base (*12*). Only the first step in the polar mechanism is feasible with CPG as the substrate (*13*), a feature that should facilitate detection of a putative 4a-adduct intermediate. Single electron transfer (SET) mechanisms are initiated by a one-electron transfer from the substrate amino group to the flavin. The amine cation radical formed upon one-electron oxidation of CPG via a SET mechanism is expected to undergo rapid cyclopropyl ring opening ($k > 5 \times 10^8$ s^{-1}) (*14*) to yield a reactive radical that might covalently modify MSOX and cause enzyme inactivation.

† This work was supported in part by Grant GM 31704 (M.S.J.) from the National Institutes of Health and NSF Grant MCB-0343374 (F.S.M.).

‡ Crystallographic coordinates have been deposited in the Protein Data Bank under the file name 2A89.

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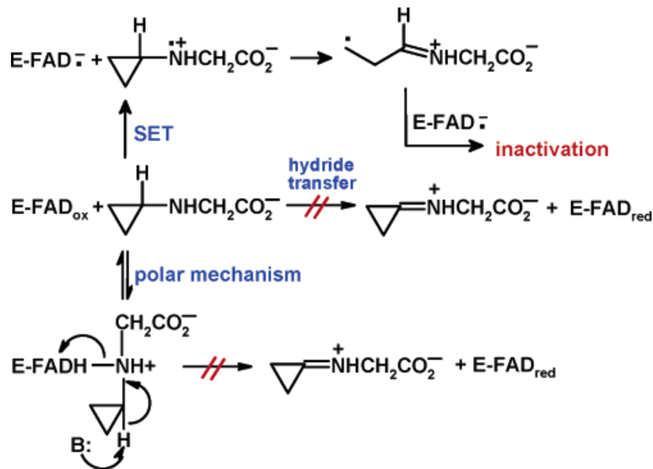
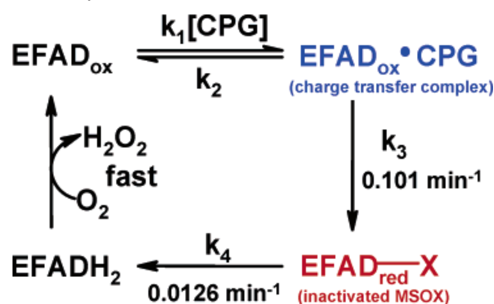
1 Abbreviations: MSOX, monomeric sarcosine oxidase; FAD, flavin adenine dinucleotide; CPG, *N*-(cyclopropyl)glycine; SET, single electron transfer; rmsd, root-mean-square deviation.

10.1021/bi0515422 CCC: $30.25 © 2005 American Chemical Society
Published on Web 11/05/2005

Scheme 1: Possible Reactions of MSOX with CPG



Scheme 2: Kinetic Mechanism Proposed for MSOX Modification by CPG[a]



[a] For this mechanism, the extent of modification under aerobic conditions at steady-state can be estimated using values determined for k_3, k_4, K_i [$K_i = (k_2 + k_3)/k_1$ = 87 mM] and eq 1 (*1*): ([EFAD$_{red}$-X]/[E$_{total}$]) = (k_3[CPG])/(k_4K_i + [CPG](k_3 + k_4)).

We previously found that CPG acts as a mechanism-based inactivator of MSOX and obtained substantial evidence for the *kinetic* mechanism shown in Scheme 2 (*1*). When MSOX is treated with excess CPG a charge transfer complex is immediately formed which exhibits an intense long-wavelength absorption band in the 500−700 nm range. The charge transfer complex reacts to form a covalently modified reduced flavin of unknown structure (EFAD$_{red}$−X) [λ_{max} = 410 and 320 nm, (ϵ_{410} = 2980 M^{-1} cm^{-1})][2] (Figure 1), accompanied by a loss of enzyme activity. The CPG-modified flavin is converted at an 8-fold slower rate to 1,5-dihydroFAD (EFADH$_2$). When the reaction is conducted under aerobic conditions, EFADH$_2$ is rapidly oxidized to yield unmodified oxidized enzyme (EFAD$_{ox}$), which can initiate a second round of reaction with CPG. As a result, CPG-modified MSOX reaches a CPG-dependent steady-state concentration under aerobic conditions. Nearly complete inactivation is observed in the presence of excess CPG under aerobic conditions. However, removal of CPG by dialysis or dilution under aerobic conditions shifts the steady state

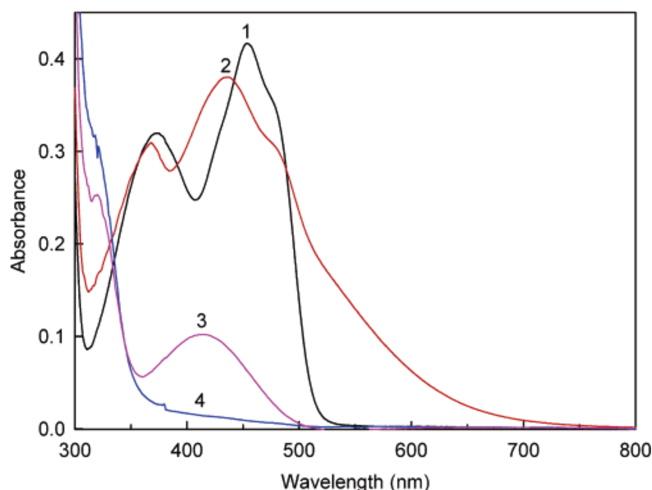


FIGURE 1: Spectral properties of various forms of MSOX. Curve 1 is the absorption spectrum of free unmodified MSOX. Curve 2 is the spectrum of the charge-transfer complex with CPG (EFAD$_{ox}$•CPG), recorded immediately after mixing with 540 mM CPG. Curve 3 is the spectrum of CPG-modified MSOX. The spectrum was recorded 90 min after mixing with 540 mM CPG and has been corrected for the presence of 11.0% EFAD$_{ox}$•CPG at steady state. Curve 4 is the spectrum of borohydride-reduced CPG-modified enzyme. All spectra were recorded with 34.2 μM MSOX in aerobic buffer (50 mM potassium phosphate, pH 8.0) at 25 °C.

in favor of unmodified oxidized enzyme (EFAD$_{ox}$). This reaction proceeds via an EFADH$_2$ intermediate that is detected when aerobically formed CPG-modified flavin is diluted into CPG-free anaerobic buffer. No loss of MSOX activity is observed when the reaction with CPG is initiated under anaerobic conditions in which all of the enzyme is slowly converted to EFADH$_2$.

CPG-modified MSOX can be further reduced and stabilized by treatment with sodium borohydride in a reaction that results in the loss of the absorption band at 410 nm (Figure 1). Borohydride-reduced CPG-modified MSOX does not revert to its unmodified oxidized flavin form upon dialysis and remains completely inactive (*1*).

In this paper, we present mass spectral and crystallographic analysis of borohydride-reduced CPG-modified MSOX. The crystal structure, determined at 1.85 Å resolution, contains a fused five-membered ring linking the C(4a) and N(5) atoms of the flavin ring as well as a tetrahedral carbon atom at flavin C(4) with a hydroxyl group attached to C(4).

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Reaction with CPG. Recombinant MSOX was prepared as described by Wagner et al. (*2*). Enzyme concentration was estimated based on its absorbance at 454 nm (ϵ_{454} = 12 200 M^{-1} cm^{-1}) (*2*). Borohydride-reduced CPG-modified enzyme for mass spectral and crystallographic analysis was prepared by reaction with 200 mM CPG, as described previously (*1*).

Mass Spectrometry. Samples of unmodified MSOX and borohydride-reduced CPG-modified MSOX were prepared as previously described (*1*) and then exchanged into 10 mM ammonium acetate pH 8.0. Just prior to analysis, the samples were denatured with 50% methanol in water containing 1% acetic acid. Electrospray mass ionization (ESI) spectral analysis was performed using a 7 T Fourier Transform Ion

[2] Previously reported spectral properties for CPG-modified MSOX (λ_{max} = 422 nm, shoulder at 317 nm) were based on data obtained for the aerobic reaction with excess CPG (*1*) but were not corrected for the presence of EFAD$_{ox}$•CPG at steady state. The spectrum of CPG-modified MSOX shown in Figure 1 is corrected for the presence of 11.0% EFAD$_{ox}$•CPG at steady state, as judged by residual absorption at 519 nm due to the charge-transfer complex. The observed value (11.0%) is in good agreement with a calculated value (12.6%) based on the kinetic mechanism shown in Scheme 2.

Cyclotron Resonance (FTICR) mass spectrometer at the Pacific Northwest National Laboratory.

Crystallization and Data Collection. Crystals of CPG-modified MSOX were grown by the sitting drop vapor-diffusion method as described previously (*8*). Five microliters of protein solution at 10 mg/mL in 20 mM Tris-HCl buffer, pH 8.0, and 5 μL of reservoir solution containing 2.0−2.2 M Na/K phosphate buffer, pH 6.7, were mixed and left to equilibrate at 23 °C. Single crystals grew to an approximate size of 0.4 × 0.4 × 0.25 mm^3 in 1−2 weeks. The crystals are monoclinic, space group $P2_1$, with unit cell parameters $a = 72.3$ Å, $b = 69.1$ Å, $c = 74.1$ Å, and $\beta = 94.1°$. There are two molecules in the asymmetric unit.

X-ray data were recorded to 1.85 Å resolution from a single-crystal soaked 7 min in paratone-N oil at 100 K on a Rigaku R-axis IV image plate detector using a Ni-filtered mirror focused X-ray beam obtained from a Rigaku RU200 X-ray generator operated at 5 keV power. Data processing including indexing, integrating, and scaling was performed using the HKL package (*15*). The data are 89.2% complete in the resolution range 40−1.85 Å and the overall R_{merge} value[3] is 6.8%. The results of data collection are shown in Table 1.

Structure Solution and Refinement. The crystal structure of CPG-modified MSOX was refined directly using CNS (*16*) starting with the model of native MSOX, PDB code 1L9F, with FAD included, but with solvent molecules and alternate conformers omitted. A total of 10% of the reflections were selected randomly and set aside as a test set for cross validation (*17*). Noncrystallographic symmetry (NCS) restraints were applied with NCS weights set to 300 for both main and side atoms except for two short polypeptide segments plus four side chains, all near the protein surface and which consistently differed from each other in the two molecules. After an initial round of rigid-body refinement was carried out at 2.5 Å resolution, followed by positional, slow cooling annealing and B-factor refinement, the refinement was gradually extended to 1.85 Å resolution. A few rounds of minimization, including bulk solvent correction, B-factor refinement and model rebuilding, gave $R_{work} = 0.245$ and $R_{free} = 0.274$ values[3] at 1.85 Å resolution. Thereafter, water molecules were added interactively at the end of each refinement cycle to peaks over 3σ in height in the $F_o - F_c$ electron density maps. The model of CPG-modified FAD was obtained during the final refinement stage from the F_o-F_c and $2F_o - F_c$ electron density maps as described below. The final R_{work} and R_{free} values were 0.178 and 0.221, with root-mean-square deviation (rmsd) from ideal values of 0.007 Å for bond lengths and 1.42 ° for bond angles, respectively. The refinement and model parameters are listed in Table 1.

Modeling of Borohydride-Reduced CPG-Modified FAD. The nearly final $F_o - F_c$ and $2F_o - F_c$ electron density difference maps in the two independent subunits that were calculated using unmodified FAD (i.e., before modeling the CPG adduct) were quite similar and showed density protruding from the *re* face of the flavin ring density at C(4a) and C(5) and extending in front of N(5) (Figure 2A). To satisfy this density and other distorted features of the flavin ring, a model of 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin (*18*) (Cambridge Structural Database entry HEHLFB10), was superimposed on the flavin ring of FAD using the molecular graphics package Turbo-Frodo (*19*). This model compound contains a five-membered ring fused to the flavin ring. Some of the atoms were changed and others were added to match the chemical composition expected from the reaction, as will be discussed. The pucker of the five-membered ring was then adjusted to match that of D,L-proline using as the model the structure IUCr reference: HU1113 (*20*). Further modeling of the borohydride-reduced CPG-modified FAD was then carried out using the program CNS (*16*). 200 cycles of energy-minimization were followed by Cartesian molecular dynamics simulation (integration time 0.0005 ps, 1000 steps, 298 K) and 200 cycles of additional energy-minimization to give the final model.

[3] The definitions of R_{merge}, R_{work}, and R_{free} are given in the legend to Table 1.

Table 1: Summary of Data Collection and Refinement for Borhydride-Reduced CPG-Modified MSOX

Data Collection	
space group	$P2_1$
unit cell dimensions (Å)	$a = 72.3$, $b = 69.1$, $c = 74.1$
(°)	$\beta = 94.1$
no. mol. per asymmetric unit	2
resolution range (last shell) (Å)	40−1.85 (1.92−1.85)
number of observations	214 783
unique observations	55 726
completeness (last shell) (%)	89.2 (49.4)
R_{merge}[a] (last shell) (%)	6.8/25.4
$I/\sigma(I)$[b] (last shell)	12.4 (2.4)
Refinement	
resolution (Å)	40−1.85
$\|F\|/\sigma(\|F\|)$	>0
R_{work}[c]	0.178
R_{free}[d]	0.221
reflections (working/test)	49971/5673
no. of protein/FCG[e] atoms	6018/114
solvent molecules/other atoms[f]	980/8
rmsd bond lengths[g] (Å)	0.007
rmsd angles[g] (°)	1.42
rms ΔB ($Å^2$) (mm/ms/ss)[h]	1.93/2.44/3.47
$\langle B\rangle$ protein ($Å^2$)	15.1
$\langle B\rangle$ FCG[e] ($Å^2$)	9.8
$\langle B\rangle$ solvent ($Å^2$)	25.8
$\langle B\rangle$ other atoms[f] ($Å^2$)	17.4
Ramachandran plot (%)	
allowed region	99.7
generously allowed region	0
disallowed region	0.3

[a] $R_{merge} = \sum_h\sum_i I(h) - I_i(h)/\sum_h\sum_i I_i(h)$, where $I_i(h)$ and $I(h)$ are the *i*th and mean measurements of reflection *h*. [b] $I/\sigma(I)$ is the average signal-to-noise ratio for merged reflection intensities. [c] $R_{work} = \sum_h(F_o - F_c)/(\sum_h F_o)$, where F_o and F_c are the observed and calculated structure factor amplitudes for the working set of reflections *h* used for the refinement. [d] R_{free} is same expression as R_{work} and refers to the test reflection data set, about 10% selected randomly for cross validation during crystallographic refinement (*28*). [e] FCG is the FAD cofactor after modification by CPG and then stabilization by borohydride reduction. [f] Other atoms, including one PO_4^{3-} and three Cl^- ions. [g] Root-mean-squared deviation (rmsd) from ideal bond lengths and angles and rmsd in B-factors of bonded atoms. [h] mm, main chain to main chain; ms, main chain to side chain, ss, side chain to side chain.

RESULTS

Mass Spectrometry. Borohydride-reduced CPG-modified MSOX exhibits a mass increase of 63 ± 2 Da as compared with unmodified MSOX, as judged by electrospray mass spectral analysis under denaturing conditions (Figure 3). A chemical mechanism for the MSOX reaction with CPG that

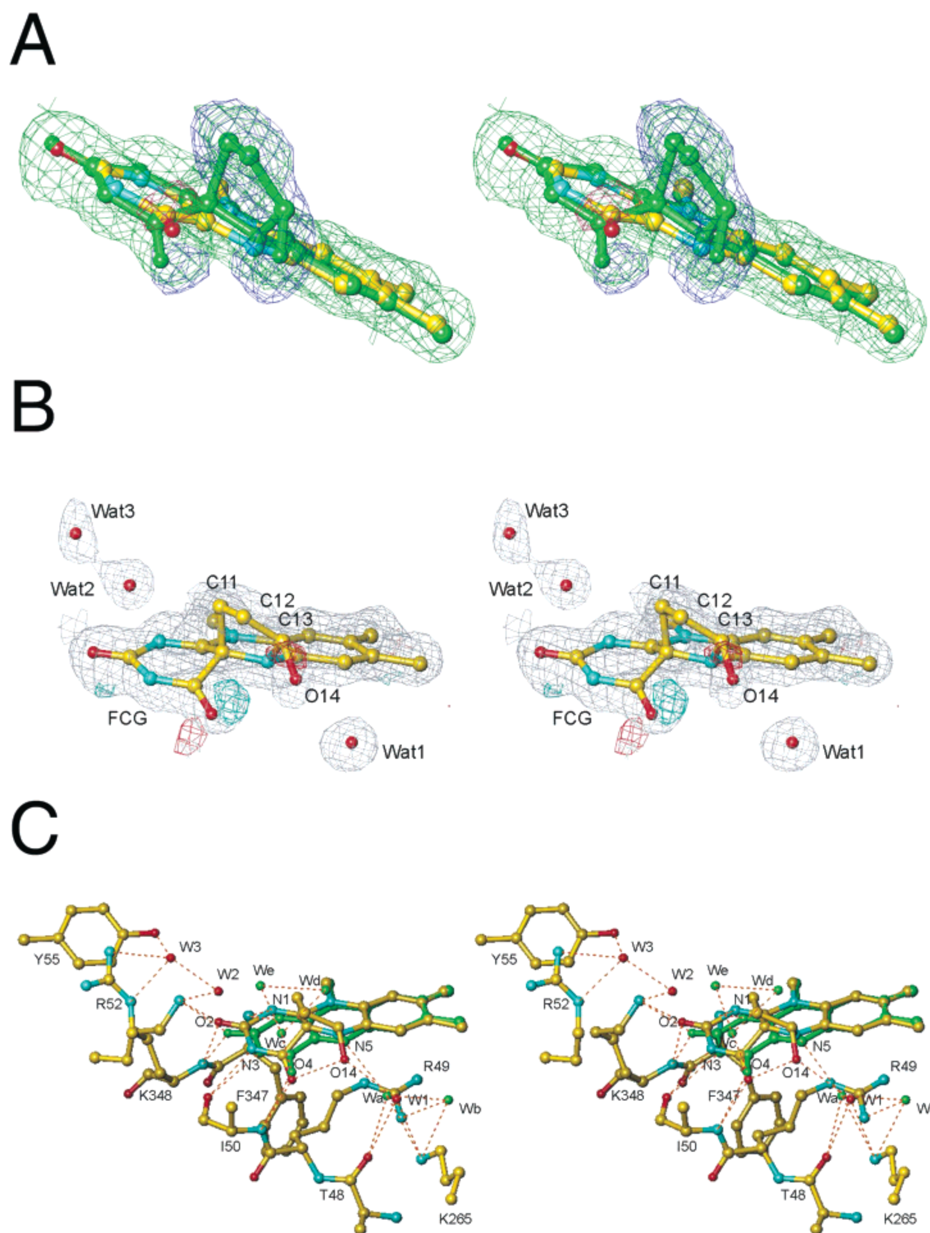


FIGURE 2: Molecular diagrams of borohydride-reduced CPG-modified MSOX. (A) The $2F_o - F_c$ (green, contour level 1 σ) and $F_o - F_c$ (blue and red, contour levels $+3\sigma$ and -3σ, respectively) difference electron density maps, computed using unmodified FAD in the model, are shown. The isoalloxazine ring of FAD, shown with its atoms colored carbon yellow, nitrogen cyan, and oxygen red, and the CPG-modified isoalloxazine ring colored green, are superimposed on the density. (B) The final $2F_o - F_c$ electron density map (gray, contour level 1 σ) and the $F_o - F_c$ difference map (cyan and red, contour levels $+3\sigma$ and -3σ, respectively) are shown with the borohydride-reduced CPG-modified isoalloxazine ring (labeled as FCG) superimposed. Three water molecules are also shown. Atom colors are as in panel A for FAD. The four new atoms of the adduct (C11−C13, O14) are labeled. Panel C shows a superposition of the stick models of native (green) and borohydride-reduced CPG-modified MSOX (atom colors as in panels A and B). The hydrogen-bonding interactions of each of the models and of their associated water molecules (wata-wate of native MSOX, wat1-wat3 of modified MSOX) with surrounding protein side chains are also shown. This diagram was made using Turbo-Frodo (*19*).

is consistent with the observed flavin modification and the mass spectral data is suggested in Scheme 3. We postulate that the reaction is initiated by a SET step that generates a flavin/CPG radical pair. Rapid opening of the cyclopropyl ring, followed by addition of the ring-cleaved CPG radical to flavin C(4a), yields compound 1 ($\Delta m = 115$ Da), analogous to the product formed by a SET mechanism in a photochemical model flavin reaction with a cyclopropylamine (*21*). Hydrolysis of the imine linkage in compound 1 yields compound 2, a 4a,5-dihydroflavin with a substituent at C(4a) containing three carbon atoms and one oxygen atom ($\Delta m = 58$ Da). Compound 2 (or compound 1) can undergo a base-catalyzed retro-Michael reaction to yield unmodified 1,5-dihydro-FAD, consistent with the observed instability of the CPG-modified flavin. Compound 2 may exist in equilibrium with a cyclic derivative, compound 3, formed by nucleophilic attack of flavin N(5) on the aldehyde group in the C(4a) substituent. Borohydride should reduce the C(10a)-N(1) imine function in compound 2 or 3, as judged by results obtained for related 4a,-5-dihydroflavin derivatives (*22*). This reaction alone would be sufficient to stabilize the CPG-modified flavin. The reagent will, however, also reduce the aldehyde group in compound 2. Our previous studies with unmodified MSOX (*23*) strongly suggest that the carbonyl

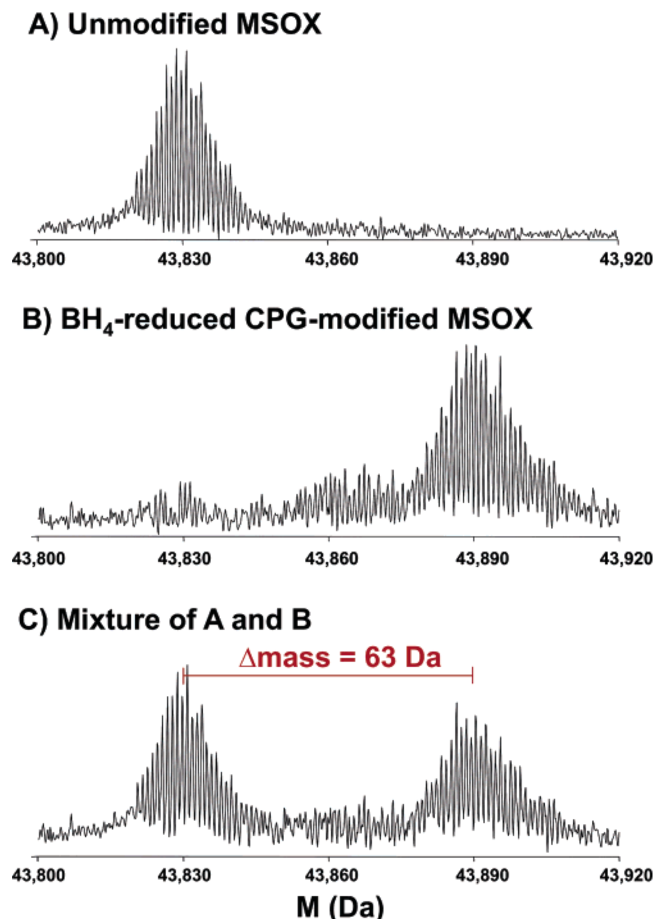


FIGURE 3: Deconvoluted ESI-FTICR mass spectral data obtained for unmodified MSOX (A), borohydride-reduced CPG-modified MSOX (B), and a mixture of the two enzyme forms (C).

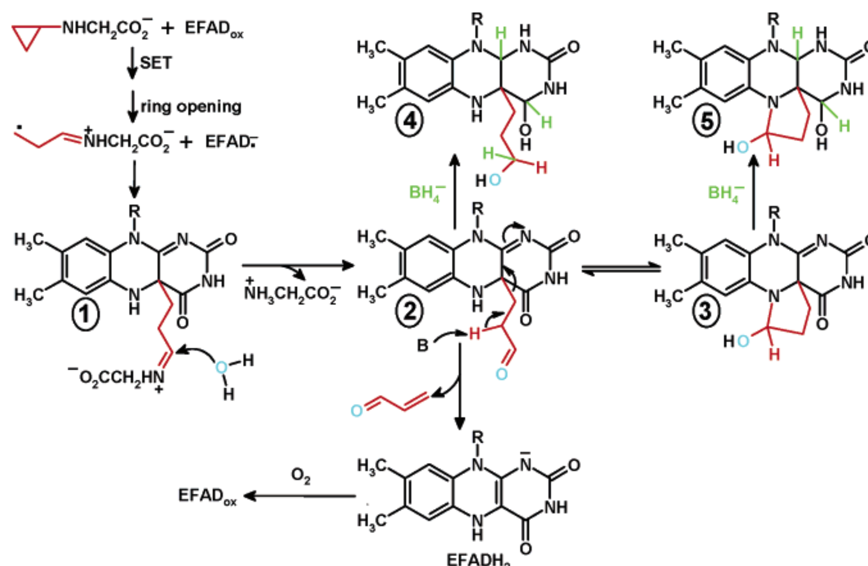
group at flavin C(4) in compounds 2 and 3 will also be reducible by borohydride. These considerations led us to predict two possible structures for borohydride-reduced CPG modified MSOX: compounds 4 (Δm = 64 Da) and 5 (Δm = 62 Da). Both structures are compatible with the mass spectral analysis (Δm = 63 ± 2 Da).

Structure Analysis. Evidence to evaluate the postulated chemical mechanism for CPG modification was sought by determining the X-ray structure of borohydride-reduced CPG-modified MSOX. Crystals of the modified enzyme were obtained that diffracted to 1.85 Å. The structure of the CPG-modified MSOX is, overall, very similar to that of the native enzyme, consistent with a high degree of isomorphism with the latter, and the relative ease of directly refining the native structure against the observed structure factors from the CPG derivative. The largest discrepancy in the $F_o - F_c$ electron density difference maps was in the flavin ring, with unexplained positive features near the C(4a) and N(5) atoms and negative features close to certain ring atoms suggestive of nonplanarity of the ring. Of great utility for the model building were the results of mass spectroscopic studies which indicated an increase in the mass of the derivatized enzyme of 63 ± 2 mass units. This was consistent with the addition of four non-hydrogen atoms (C, N, or O) to the protein. After various model building trials, we concluded that the $F_o - F_c$ density could be explained best by three carbon atoms connecting C(4a) and N(5) of the flavin ring, to give a fused five-membered ring, with an additional atom being bound to the atom attached to N(5), and a tetrahedral configuration at flavin C(4) suggesting O4 to be a hydroxyl group.

A model for the reaction product of CPG modification of the flavin ring followed by borohydride reduction of that product, corresponding to compound 5 of the proposed mechanism for product formation (Scheme 3), was then constructed on the graphics system. The published structure of 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin (*18*) served as a useful template for the initial modeling. An appropriate pucker for the five-membered ring to fit the omit and difference electron density was then introduced, based on the structure of D,L-proline (*20*), and the energy of the resulting model was minimized using CNS (*16*). The final model of the borohydride-reduced CPG-modified isoalloxazine ring is compared to the electron density in Figure 2B.

Structure of the Borohydride-Reduced CPG-Modified MSOX Flavin (CPG-Adduct). In addition to the FAD isoalloxazine ring, the CPG-adduct has three carbon atoms, one oxygen atom, and 10 hydrogen atoms (Scheme 3, compound 5). The three carbon atoms (C11−C13) connect flavin atoms C(4a) and N(5) to form a five-membered ring,

Scheme 3: Proposed Chemical Mechanism for the Reaction of MSOX with CPG and Borohydride Reduction of the CPG-Modified Flavin



and the oxygen is appended to C13 as a hydroxyl group. The five-membered ring contains five (additional) hydrogens attached to C11−C13. Four hydrogens are added to flavin C(10a), C(4), N(1), and O(4) (as a hydroxyl group). The CPG-adduct and three water molecules (Wat1-Wat3) replace the unmodified FAD plus five water molecules (Wata-Wate) found in native MSOX (Figure 2C). In the CPG-adduct, flavin O(4) forms a hydrogen bond to O14 (attached to C13 in the five-membered ring). In both the CPG-adduct and FAD in native MSOX, flavin O(4) receives a proton from Ile50 N. Wat1 in the modified enzyme receives a proton from O14 in the CPG-adduct, whereas the corresponding water in native enzyme (Wata) donates a proton to flavin N(5). Watb in native MSOX, which donates a proton to Wata and receives protons from Arg49 and Lys265 side chains, is absent in the modified enzyme. Wat1 in modifed enzyme and Wata in native MSOX are both hydrogen bonded to Lys265 N^{ζ} and to Thr48O. Watc in native MSOX forms a hydrogen bond to flavin O(4), but it, along with Watd and Wate, are displaced in the modified enzyme by the additional atoms in the CPG-adduct. Flavin N(3) and O(2) make the same hydrogen bonds in both native and modified enzyme (with Ile50 O and with Lys348 N and N^{ζ}, respectively). Flavin N(1) in both structures appears to interact weakly with Phe347N (3.2−3.3 Å), but it is not clear whether a hydrogen bond is formed. Finally, Wat2 and Wat3 are introduced above the flavin ring in the CPG-adduct (at positions that are vacant in native MSOX) and form hydrogen bonds with Lys348, Arg52, Tyr55, and each other.

DISCUSSION

The crystal structure of borohydride-reduced CPG-modified MSOX was solved at 1.85 Å resolution. The results show that the FAD is only site of modification. The modified FAD contains a fused five-membered ring, linking the C(4a) and N(5) atoms of the flavin ring, with an additional hydroxyl group bound to the atom attached to N(5), and a tetrahedral carbon atom with an attached hydroxyl group at position C(4). A model for the modified FAD corresponding to compound 5 in Scheme 3 was developed and found to give a good fit to the observed electron density (Figure 2). The only consistent deviations in the two independent molecules in the asymmetric unit are a small negative difference peak between C13 and C14 and a positive/negative difference pair adjacent to O(4). These peaks probably represent minor discrepancies in the minimization procedure and the limited resolution of the X-ray data. There is no indication of the presence in the crystal of a significant amount of the noncyclic form of the borohydride-reduced CPG-modified flavin, compound 4 of Scheme 3. However, the possibility that both compounds 4 and 5 are formed in solution, but only compound 5 selectively crystallized, cannot be ruled out.

On the basis of the crystal structure of the borohydride-stabilized adduct, we conclude that the labile CPG-modified flavin is a 4a,5-dihydroflavin derivative that may exist as a mixture of acyclic and cyclic isomers (Scheme 3, compounds 2 and 3, respectively). That the CPG-modified flavin is a dihydro derivative is consistent with its conversion to 1,5-dihydroFAD, a reaction observed with either intact or denatured enzyme (*1*). 4a,5-Dihydroflavins are known to be reduced by borohydride, yielding 4a,5,10a,1-tetrahydroflavins that exhibit spectral properties similar to that observed for the borohydride-reduced CPG-modified flavin (*22*). CPG-modified MSOX exhibits two absorption maxima at $\lambda > 300$ nm ($\lambda_{max} = 410$ and 320 nm), similar to that reported for related 4a,5-dihydroflavin derivatives (e.g., 3-methyl-4a,5-propano-4a,5-dihydrolumiflavin, $\lambda_{max} = 386$, 313 nm) (*24*) except for a bathochromic shift of the lowest energy absorption band. The reason for this difference is unclear.

Inactivation of monoamine oxidase with *trans*-2-phenylcyclopropylamine results in the formation of a 4a-substituted 4a,5-dihydroflavin (*25*), analogous to the acyclic form of the modified flavin proposed for the MSOX reaction with CPG (Scheme 3, compound 2). No evidence for the corresponding cyclic form was found with inactivated monoamine oxidase, suggesting that the bulky phenyl substitutent and/or other steric constraints may interfere with the cyclization reaction. Although borohydride reduction was not required to stabilize the modified flavin in monamine oxidase, its lability was apparent by the appearance of oxidized flavin upon aerobic denaturation of the inactivated enzyme. Significantly, there is no base at the active site of monoamine oxidase, unlike MSOX where His269 might facilitate the postulated retro-Michael reaction with the CPG-modified flavin that triggers its conversion to unmodified flavin (Scheme 3).

The observed CPG-mediated inactivation of MSOX, accompanied by cleavage of the cyclopropyl ring, is consistent with the SET mechanism proposed in Scheme 3. An alternate ring cleavage mechanism was suggested by Mariano and co-workers based on model studies with cyclopropylamines and a flavin derivative activated toward nucleophilic addition at C(4a) (*13*). Mariano observed rapid formation of a relatively stable 4a-cyclopropylamine adduct, as expected in the polar mechanism for amine oxidation (Scheme 1) when the second step (α-hydrogen abstraction) is not feasible. A base-catalyzed ring opening reaction, possibly initiated by abstraction of the β-hydrogen, was observed but only after prolonged incubation of the 4a-cyclopropylamine adduct at elevated temperature (7 days, 85 °C). The Mariano mechanism is unlikely to account for the reaction observed with MSOX and CPG where the first step involves rapid formation of a charge-transfer complex, not a C(4a)-adduct. Edmondson and co-workers (*25*) have suggested that inactivation of monoamine oxidase by *trans*-2-phenylcyclopropylamine can occur via a non-SET mechanism "similar to that shown by Sayre's laboratory for quinone-mediated oxidative cleavage of cyclopropylamine" (*26*) but provided no details or information in support of this puzzling proposal.

The first step in the reaction of MSOX with CPG involves formation of a charge transfer complex. Interestingly, a similar feature is observed with normal (nonsuicide) substrates. We have previously shown that MSOX binds the L-proline zwitterion, induces a large decrease in the pK_a of the bound amino acid to generate the reactive, electron-rich L-proline anion which acts as the charge transfer donor (*9, 27*). Although L-proline is a slow substrate for MSOX, recent rapid reaction studies indicate that a charge-transfer complex is also formed as the first step in the reaction of the enzyme with sarcosine.[4] Although the results obtained with CPG

[4] Zhao, G., and Jorns, M. S., unpublished results.

indicate that MSOX is apparently capable of amine oxidation via a SET mechanism, the slow rate of the reaction suggests that additional evidence is needed before extrapolation of these results to the physiological reaction.

ACKNOWLEDGMENT

We thank Dr. Lawrence Sayre and Amy Kaufman for helpful discussions. The ESI-FTICR mass spectral studies were performed at the W. R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the U.S. Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory. PNNL is operated for the Department of Energy by Battelle.

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BI0515422