Articles

Tautomeric Rearrangement of a Dihydroflavin Bound to Monomeric Sarcosine Oxidase or *N*-Methyltryptophan Oxidase†

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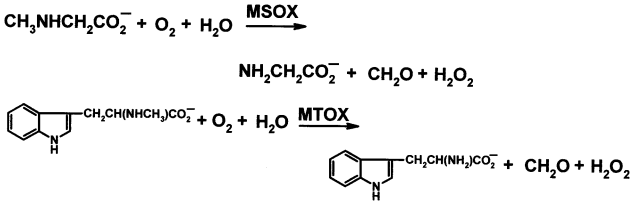
ABSTRACT: Monomeric sarcosine oxidase (MSOX) and *N*-methyltryptophan oxidase (MTOX) are homologous bacterial flavoenzymes that contain covalently bound flavin [8α-(*S*-cysteinyl)FAD]. Reaction of MSOX or MTOX with a small excess of sodium borohydride results in immediate flavin reduction to a species that exhibits spectral properties (λ_{max} = 405 nm with a second broad peak at 332 nm) similar to those of 3,4-dihydroflavin. The borohydride-reduced enzymes retain full catalytic activity. Substrate reduction converts the 405 nm species to an air-sensitive tetrahydroflavin that reacts with oxygen to yield unmodified oxidized enzyme. Unexpectedly, the putative 3,4-dihydroflavin bound to MSOX or MTOX is unstable in the absence of substrate. An isosbestic conversion of the 405 nm species to yield unmodified, oxidized flavin is observed when the reaction is conducted under aerobic conditions ($k_{obs} = 4.9 \times 10^{-2}$ min^{-1}). Under anaerobic conditions, an oxygen-sensitive species resembling 1,5-dihydroflavin is formed in an isosbestic reaction that occurs at a rate similar to that of the aerobic reaction ($k_{obs} = 5.3 \times 10^{-2}$ min^{-1}). Possible reaction of the 3,4-dihydroflavin with a second molecule of borohydride to yield an air-sensitive tetrahydroflavin is unlikely since prior scavenging of residual borohydride with excess formaldehyde had no effect on the aerobic conversion to unmodified oxidized flavin. The observed instability is attributed to a tautomeric rearrangement of the 3,4-dihydroflavin to generate 1,5-dihydroflavin, a species that is also air-sensitive. Evidence in favor of an active site facilitated tautomerization reaction is provided by the fact that the stability of the 405 nm species formed with MSOX is enhanced 200-fold upon denaturation with urea or heat. The observed tautomeric rearrangement of 3,4-dihydroflavin may provide insight regarding a related flavin tautomerization reaction that has been proposed as a key step in the biosynthesis of covalent flavin linkages.

Monomeric sarcosine oxidase (MSOX)[1] and *N*-methyltryptophan oxidase (MTOX) are bacterial flavoenzymes that exhibit considerable amino acid sequence identity (43%), contain covalently bound flavin [8α-(*S*-cysteinyl)FAD], and catalyze similar oxidative demethylation reactions, albeit with dramatically different substrate specificity as judged by the preferred *N*-methyl amino acid substrates (glycine versus tryptophan) (*1*−*5*).

N-(Cyclopropyl)glycine (CPG) acts as a suicide substrate for MSOX in a reaction that results in covalent modification of the flavin. The CPG-modified flavin is labile but can be stabilized by reduction with borohydride (*6*). The borohydride-reduced CPG-modified enzyme has been crystallized.



Preliminary analysis indicates that C(4) of the flavin is tetrahedral and a substituent has been introduced at C(4a).[2] Since the apparent reduction of the flavin at C(4) was not an expected outcome of CPG inactivation, we considered the possibility that the carbonyl group at C(4) in unmodified MSOX (or MTOX) might be susceptible toward reduction by borohydride, as previously observed with D-amino acid oxidase (DAAO) and L-amino acid oxidase (LAAO) but not with most other flavoproteins (*7*, *8*). In this paper we describe the reaction of borohydride with MSOX and MTOX and the unexpected instability of the reaction product.

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[1] Abbreviations: MSOX, monomeric sarcosine oxidase; MTOX, *N*-methyltryptophan oxidase; CPG, *N*-(cyclopropyl)glycine; FAD, flavin adenine dinucleotide; DAAO, D-amino acid oxidase; LAAO, L-amino acid oxidase.

[2] Z. Chen, G. Zhao, M. S. Jorns, and F. S. Mathews, unpublished results.

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EXPERIMENTAL PROCEDURES

Materials. Guanidine hydrochloride was from Fisher. *N*-Methyl-L-tryptophan, urea, and sodium borohydride were obtained from Sigma. Recombinant DAAO from pig kidney was a generous gift from Dr. Retsu Miura (Kumamoto University School of Medicine).

Methods. Recombinant forms of MSOX and MTOX were purified as previously described (*5*). Absorption spectra were recorded using a Perkin-Elmer Lambda 2S spectrometer. All spectra are corrected for dilution. Anaerobic experiments were conducted as previously described (*1*). Reaction kinetic data were fit to a single-exponential expression ($A_{obs} = \Delta A_{max}e^{-kt} + A_{final}$) using the curve fit function in Sigma Plot (Jandel Corp.). MSOX and MTOX activities were assayed as previously described (*2*, *5*).

RESULTS AND DISCUSSION

Reaction of MSOX and MTOX with Borohydride. Spectra recorded immediately after mixing MSOX or MTOX with borohydride ($\lambda_{max} = 405$ nm) are strikingly similar to that observed for the 3,4-dihydroflavin formed in the corresponding reaction with DAAO ($\lambda_{max} = 407$ nm) (Figure 1). The MSOX flavin is particularly reactive toward borohydride, as judged by the minimal amount of borohydride required for complete reaction (0.2 mM, versus 2 mM with MTOX or 30 mM with DAAO). To determine whether the covalent flavin link might contribute to the high reactivity of MSOX with borohydride, the enzyme was denatured with 3 M guanidine hydrochloride prior to reaction with borohydride. No reaction was observed with denatured enzyme upon mixing with borohydride at reagent concentrations as high as 3 mM. The reactivity of the native MSOX with borohydride is clearly dependent on the flavin active site environment.

Assays performed immediately after reaction of MSOX or MTOX with borohydride show that the reaction does not affect the catalytic activity of either enzyme, similar to that observed with DAAO and LAAO (*7*). Addition of substrate (0.5 mM *N*-methyltryptophan) immediately after aerobic reaction of MTOX with 2 mM borohydride results in the disappearance of the 405 nm species, accompanied by the transient appearance of a species with spectral properties similar to those of 1,5-dihydroflavin (Figure 2, curve 3). This reduced flavin species is then converted to unmodified, oxidized enzyme (Figure 2, curve 4) in a reaction that is virtually complete within 3.5 min. A similar reaction is observed with MSOX. The results show that the putative 3,4-dihydroflavin is reducible by substrate in a reaction that ultimately regenerates unmodified flavin, presumably because the C(4)-hydroxyl group in the tetrahydroflavin intermediate is readily oxidizable (Scheme 1). Similar results have been reported for the reaction of substrate with borohydride-treated DAAO and LAAO (*7*).

Conversion of Borohydride-Modified MSOX or MTOX to Unmodified, Oxidized Enzyme. Previous studies have shown that 3,4-dihydroflavin derivatives are stable at ambient temperature in the dark when free in solution or when bound to DAAO or LAAO in the absence of substrate (*7*−*9*). Surprisingly, the putative 3,4-dihydroflavin bound to MSOX or MTOX is not stable. An isosbestic conversion of borohydride-treated MTOX to unmodified, oxidized enzyme is observed upon incubation under aerobic conditions in the absence of substrate. The reaction exhibits apparent first-order kinetics and is complete in about 24 h ($k_{obs} = 2.9 \times 10^{-3}$ min^{-1}) (Figure 3). A similar reaction is observed with MSOX except that the MSOX reaction is more than an order of magnitude faster ($k_{obs} = 4.9 \times 10^{-2}$ min^{-1}) and complete within 2 h (Figure 4).

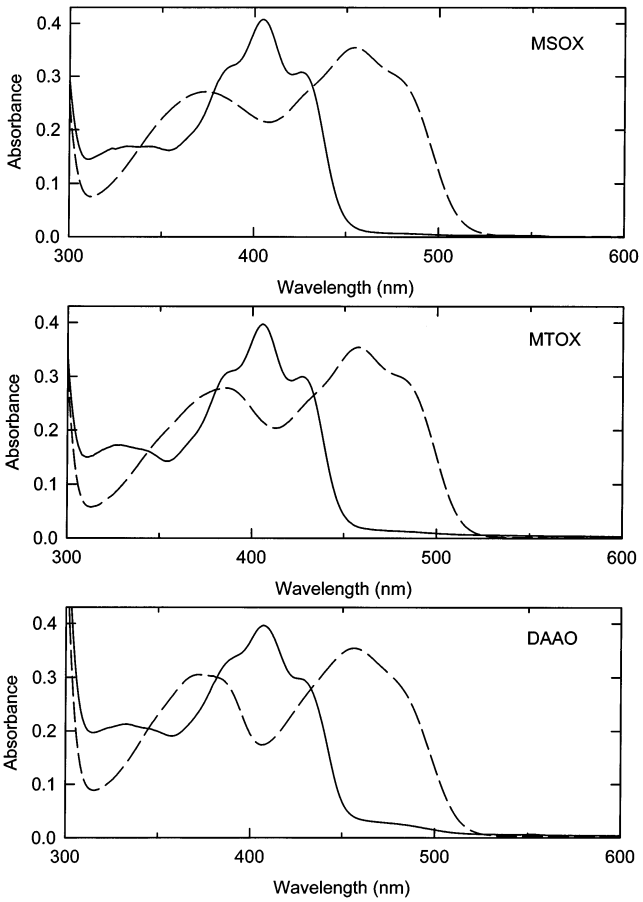


FIGURE 1: Comparison of the reactions of MSOX, MTOX, and DAAO with sodium borohydride. Reactions with MSOX, MTOX, and DAAO are shown in the upper, middle, and bottom panels, respectively. The dashed and solid lines show spectra recorded before and after reaction with borohydride, respectively. The data are normalized to the same initial absorbance at the absorption maximum of each enzyme in the 450 nm region. Spectra of borohydride-treated MSOX or MTOX were recorded immediately after addition of 0.2 or 2 mM sodium borohydride, respectively. The reactions were conducted in 50 mM potassium phosphate buffer, pH 8.0, at 25 °C; the MTOX buffer also included 1 mM EDTA. For the reaction with DAAO, borohydride was added in three equal aliquots to a final concentration of 30 mM over a period of 50 min; the spectrum shown was recorded 2 h after addition of the final aliquot. The reaction was conducted in 100 mM pyrophosphate buffer, pH 8.5, at 5 °C.

Anaerobic Reaction of MSOX with Borohydride. Insight regarding a mechanism that might account for the observed instability of the putative 3,4-dihydroflavin in MSOX and MTOX was obtained by monitoring the reaction with borohydride under anaerobic conditions. Reaction of MSOX with 0.2 mM borohydride under anaerobic conditions results in the immediate formation of the 405 nm species. Interestingly, the 405 nm species is unstable under anaerobic conditions but is not converted to unmodified, oxidized flavin, as observed for the aerobic reaction. Instead, an isosbestic conversion of the 405 nm species to a product

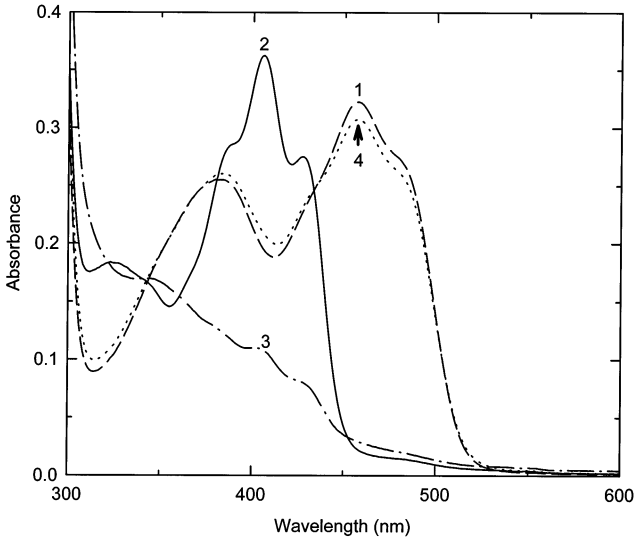
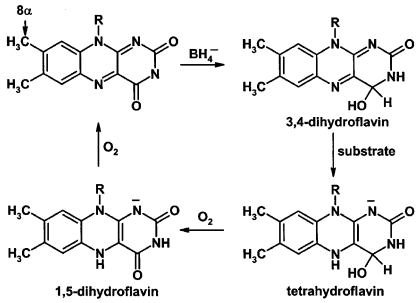


FIGURE 2: Reaction of substrate with borohydride-treated MTOX. Curve 1 (dashed line) is the initial spectrum of untreated enzyme in 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. Curve 2 (solid line) is the spectrum of borohydride-treated enzyme, recorded after mixing with 2.0 mM sodium borohydride, followed by addition of 3.6 mM KH_2PO_4 to readjust the pH to 8.0. Curve 3 (dash-dot line) was recorded immediately after addition of 0.5 mM *N*-methyl-L-tryptophan. Curve 4 (dotted line) was recorded 3.5 min after substrate addition. A spectrum recorded 23.5 min after substrate addition (not shown) superimposed with curve 1.

Scheme 1: Formation of 3,4-Dihydroflavin by Reduction with Borohydride, Followed by Substrate Reduction to an Air-Sensitive Tetrahydroflavin[a]



[a] The flavin in MSOX and MTOX is attached to a cysteine residue in the protein via a thioether link to the 8α position of the flavin ring (not shown).

with spectral properties similar to those of 1,5-dihydroflavin is observed in a reaction that exhibits apparent first-order kinetics (Figure 5). The rate of this *anaerobic* reaction (k_{obs} = 5.3 × 10^{-2} min^{-1}) is similar to that observed for the *aerobic* conversion of the 405 nm species to unmodified, oxidized enzyme (k_{obs} = 4.9 × 10^{-2} min^{-1}). When the anaerobic sample was opened to air, the reduced flavin species was immediately converted to unmodified, oxidized enzyme.

Effect of Formaldehyde on the Aerobic Stability of the 405 nm Species. Two possible mechanisms were considered that might account for the results observed for the reaction of MSOX with borohydride under anaerobic conditions. (1) The putative 3,4-dihydroflavin undergoes a rate-limiting conversion to a tetrahydroflavin that reacts rapidly with oxygen upon aeration to yield unmodified oxidized flavin, analogous to the reaction observed upon substrate reduction of borohydride-treated enzyme (Scheme 1). (2) The active

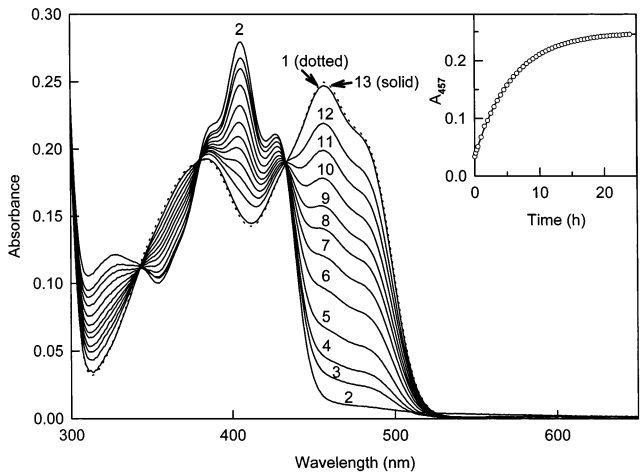


FIGURE 3: Conversion of borohydride-reduced MTOX to unmodified enzyme. Curve 1 (dotted line) is the initial spectrum of untreated enzyme in 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA at 25 °C. Curve 2 (solid line) was recorded 7 min after addition of 2 mM sodium borohydride. Curve 3 (solid line) was recorded 10 min after addition of 3.6 mM KH_2PO_4 to readjust the pH to 8.0. Curves 4−13 (solid lines) were recorded 0.33, 1, 2, 3, 4, 5, 6.5, 8.5, 11.5, and 30 h, respectively, after addition of KH_2PO_4. The inset shows a plot of absorbance changes at 457 nm versus time. The solid line shows the fit of the data (open circles) to a single-exponential expression ($A_{obs} = \Delta A_{max}e^{-kt} + A_{final}$).

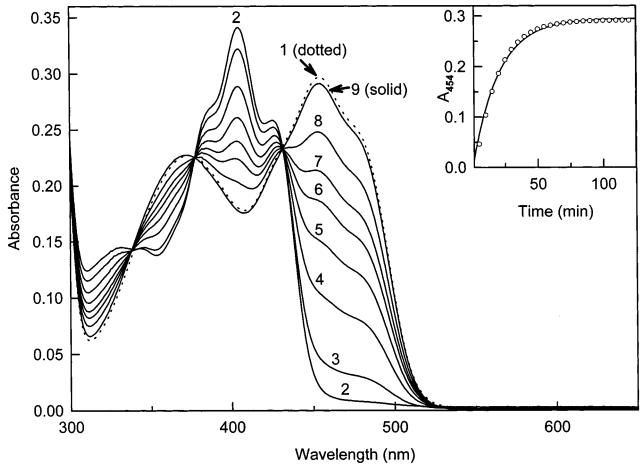


FIGURE 4: Conversion of borohydride-reduced MSOX to unmodified enzyme. Curve 1 (dotted line) is the initial spectrum of untreated enzyme in 50 mM potassium phosphate buffer, pH 8.0, at 25 °C. Curve 2 (solid line) was recorded immediately after addition of 0.2 mM sodium borohydride. Curves 3−9 (solid lines) were recorded 5, 10, 15, 20, 25, 35, and 120 min, respectively, after addition of borohydride. The inset shows a plot of absorbance changes at 454 nm versus time. The solid line shows the fit of the data (open circles) to a single-exponential expression ($A_{obs} = \Delta A_{max}e^{-kt} + A_{final}$).

site environment of MSOX (or MTOX) facilitates a rate-limiting tautomeric rearrangement of the putative 3,4-dihydroflavin to yield an oxygen-sensitive 1,5-dihydroflavin (Scheme 2a).

Mechanism 1 requires an electron donor, unlike the tautomeric rearrangement reaction in mechanism 2. The 405 nm species might be reduced by reaction with residual borohydride in solution, although the instability of the reagent in aqueous solution and the small amount used in the MSOX reaction (0.2 mM) would argue against this possibility.

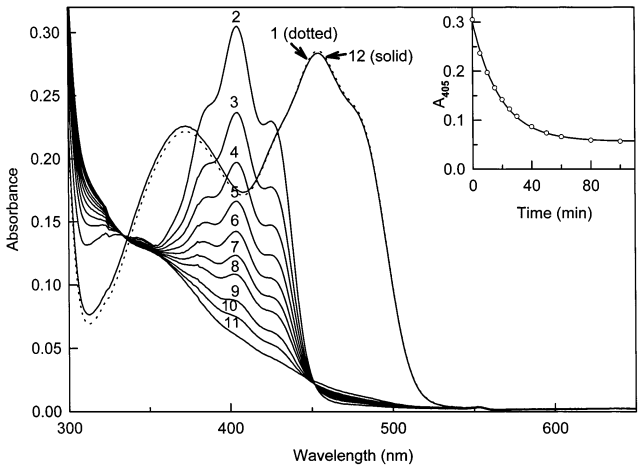
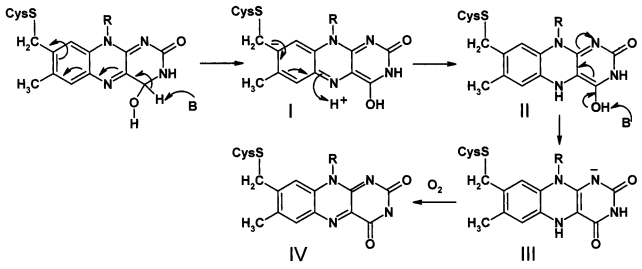


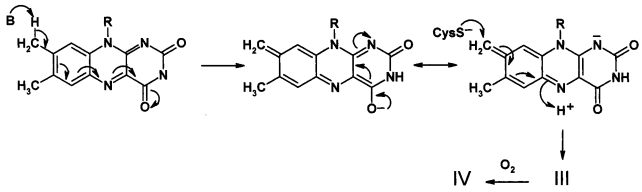
FIGURE 5: Anaerobic reaction of MSOX with sodium borohydride. Curve 1 (dotted line) is the initial spectrum of untreated enzyme in anaerobic 50 mM potassium phosphate buffer, pH 8.0, at 25 °C. Curve 2 (solid line) was recorded immediately after addition of 0.2 mM sodium borohydride. Curves 3−12 (solid lines) were recorded 5, 10, 15, 20, 25, 30, 40, 50, 60, and 80 min, respectively, after addition of borohydride. The inset shows a plot of absorbance changes at 405 nm versus time. The solid line shows the fit of the data (open circles) to a single-exponential expression ($A_{obs} = \Delta A_{max}e^{-kt} + A_{final}$).

Scheme 2[a]

Scheme 2a



Scheme 2b



[a] Scheme 2a shows the mechanism proposed for the tautomeric rearrangement of 3,4-dihydroflavin to an air-sensitive 1,5-dihydroflavin. The thioether link between the 8α position of the flavin ring and a cysteine residue in MSOX (or MTOX) is indicated. Scheme 2b shows the mechanism proposed for the covalent attachment of flavin to apoMSOX (or apoMTOX).

Definitive evidence to evaluate mechanism 1 was sought in an aerobic experiment where the 405 nm species was formed by reaction of MSOX with 0.2 mM borohydride. The sample was then immediately mixed with excess formaldehyde (1 mM) to consume any residual borohydride. The conversion of the 405 nm species to unmodified, oxidized flavin was unaffected by addition of formaldehyde. The conversion in the presence of formaldehyde occurred at a rate ($k_{obs} = 4.3 \times 10^{-2}$ min^{-1}) similar to that observed in the absence of formaldehyde ($k_{obs} = 4.9 \times 10^{-2}$ min^{-1}). The ability of formaldehyde to rapidly consume borohydride was confirmed in a control reaction where formaldehyde was mixed with MSOX just prior to the addition of borohydride. Under these conditions, borohydride had no effect on the absorption spectrum of MSOX.

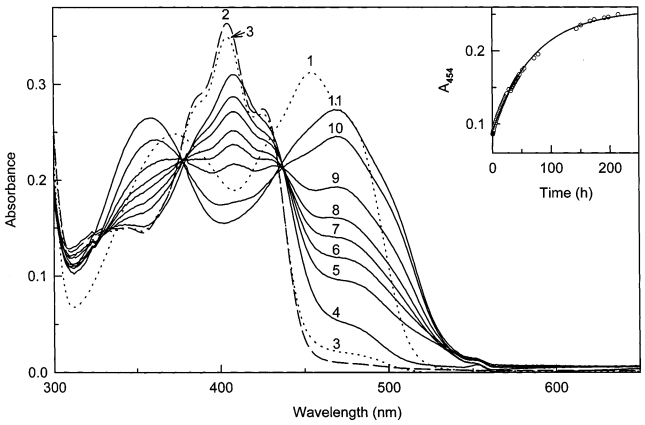


FIGURE 6: Denaturation of MSOX with urea after reaction with sodium borohydride. Curve 1 (dotted) is the initial spectrum of untreated enzyme in 50 mM potassium phosphate buffer, pH 8.0, at 25 °C. Curves 2 (dashed) and 3 (dotted) were recorded immediately after addition of 0.2 mM sodium borohydride and 1 mM formaldehyde, respectively. Curves 4−11 (solid lines) were recorded 0, 12, 21, 33.5, 44.5, 71.5, 143.5, and 414 h, respectively, after addition of 4.5 M urea. The inset shows a plot of absorbance changes at 454 nm versus time. The solid line shows the fit of the data (open circles) to a single-exponential expression ($A_{obs} = \Delta A_{max}e^{-kt} + A_{final}$).

It might be argued that conversion of the 405 nm species to an oxygen-sensitive tetrahydroflavin species involves reaction of the flavin with a molecule of borohydride sequestered at the active site. The active site of MSOX (and MTOX) should, however, be accessible to formaldehyde since the compound is generated as a product during the normal catalytic reaction of both enzymes.

Effect of Denaturation on the Stability of the 405 nm Species. Denaturation of MSOX or MTOX immediately after reaction with borohydride should interfere with an active site catalyzed tautomerization of 3,4-dihydroflavin to 1,5-dihydroflavin (Scheme 2a). This suggested that the 405 nm species might be stabilized by denaturation.

Indeed, the stability of the 405 nm species is enhanced by more than 2 orders of magnitude when MSOX is denatured with 4.5 M urea immediately after reaction with borohydride. The urea-denatured 405 nm species is not, however, completely stable. A very slow, isosbestic reaction is observed that exhibits apparent first-order kinetics ($k_{obs} = 2.3 \times 10^{-4}$ min^{-1}, $t_{1/2} = 50.2$ h) (Figure 6). The reaction is about 200-fold slower than the reaction with intact enzyme ($k_{obs} = 4.9 \times 10^{-2}$ min^{-1}). The reaction observed with urea-denatured enzyme is not only much slower but qualitatively different from that observed with the intact 405 nm species. The latter reaction yields unmodified oxidized flavin. In contrast, the reaction with the urea-denatured 405 nm species yields a modified, oxidized flavin that exhibits a 24 nm larger separation of its two visible absorption maxima ($\lambda_{max} = 359$, 468 nm), as compared with unmodified, urea-denatured MSOX ($\lambda_{max} = 368$, 453 nm), owing to bathochromic (15 nm) and hypsochromic (9 nm) shifts of the bands at longer and shorter wavelengths, respectively.

Formation of the modified, oxidized flavin upon urea denaturation of the 405 nm species is not due to a specific reaction with urea. A similar modified flavin ($\lambda_{max} = 355$, 471 nm) is formed in a slow, first-order reaction ($k_{obs} = 6.3$

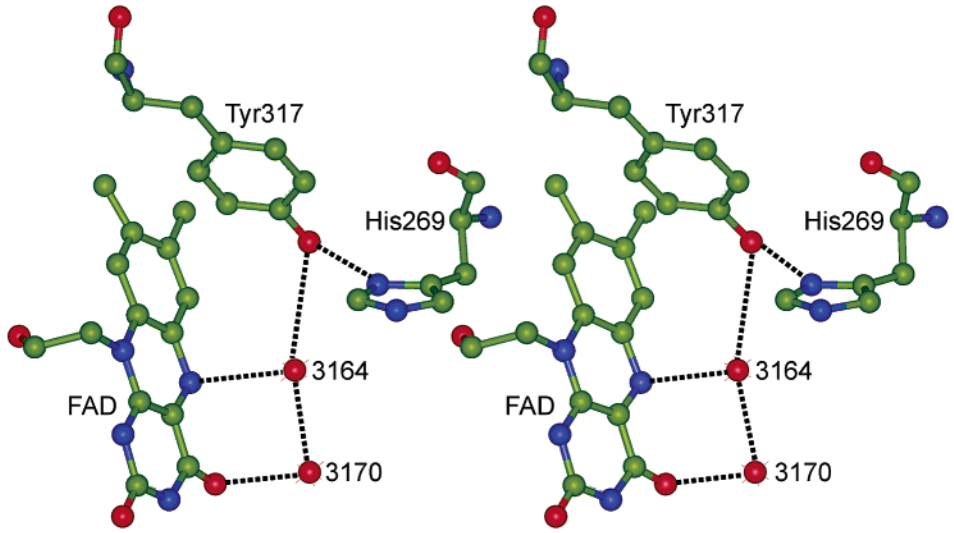


FIGURE 7: Stereoview of the active site of unmodified MSOX. The figure was constructed using the program InsightII (Accelrys) and MSOX coordinates from the Protein Data Bank (PDB code IL9F).

$\times 10^{-4}$ min^{-1}) when the 405 nm species formed with MSOX is denatured by heating at 100 °C for 1 min (data not shown). Heating does not cause visible precipitation of MSOX or interfere with spectral studies. The heat-denatured protein can, however, be pelleted by high-speed centrifugation. The modified oxidized flavin is found in the protein pellet, indicating that it still covalently attached to the protein. The modified flavin, formed after urea or heat denaturation of the 405 nm species, is readily and reversibly reduced by dithionite (data not shown), suggesting that the flavin ring is intact.

Concluding Remarks. MSOX and MTOX undergo a facile reaction immediately upon mixing with a small excess of sodium borohydride. The reaction product exhibits spectral properties (λ_{max} = 405 nm with a second broad peak at 332 nm) similar to those of the 3,4-dihydroflavin formed upon reaction of borohydride with DAAO or LAAO (*7*, *8*). The 405 nm species formed with MSOX or MTOX retains full catalytic activity. Substrate reduction of the 405 nm species yields an air-sensitive tetrahydroflavin that reacts with oxygen to yield unmodified MSOX or MTOX, similar to that observed with DAAO or LAAO. 3,4-Dihydroflavin bound to DAAO or LAAO is stable in the absence of substrate. In contrast, the putative 3,4-dihydroflavin bound to MSOX or MTOX is converted back to unmodified oxidized flavin in a reaction that proceeds via the formation of an air-sensitive 1,5-dihydroflavin intermediate. The stability of the 405 nm species formed with MSOX is enhanced 200-fold upon denaturation with urea or heat. The reaction observed with intact enzyme is postulated to involve an active site catalyzed tautomerization of 3,4-dihydroflavin to 1,5-dihydroflavin. Abstraction of a proton from the C(4) carbon in 3,4-dihydroflavin is likely to be the rate-determining step in the mechanism proposed in Scheme 2a. Although the pK_a for this reaction is not known, the resulting carbanion could be stabilized by delocalization of the negative charge into the flavin ring system, forming a quininoid flavin intermediate (I). Protonation of intermediate I at flavin N(5) yields a second intermediate (II) that can undergo a facile enol−keto tautomerization, yielding 1,5-dihydroflavin (III). Inspection of the crystal structure of MSOX does not reveal a nearby amino acid residue that could abstract a proton from flavin C(4). However, a possible proton relay is found above the *re* face of the flavin that might facilitate transfer of a proton from position C(4) to N(5). Water 3170 is located 2.6 Å from the carbonyl oxygen at C(4) and 2.8 Å from a second water (3178) that forms hydrogen bonds with flavin N(5) and the hydroxyl moiety of Tyr317, which is hydrogen bonded to His269 (Figure 7). Interestingly, intermediate I in the mechanism proposed for tautomerization of 3,4-dihydroflavin is similar to the quininoid flavin postulated as an intermediate in the flavinylation of MSOX and other enzymes containing covalently bound flavin (*10*) (Scheme 2b). This suggests that the 3,4-dihydroflavin → 1,5-dihydroflavin tautomerization reaction may be facilitated by properties of the active site that also promote autocatalytic flavinylation.

While the stability of the 3,4-dihydroflavin formed with MSOX is enhanced 200-fold upon denaturation, a very slow conversion to a modified, oxidized flavin is observed with the denatured protein. The modified flavin remains covalently attached to the protein, is reducible by dithionite, and exhibits an unusually wide separation of the two visible absorption maxima [λ_{max} = 359 and 468 nm (urea denaturation) or 355 and 471 nm (heat denaturation)]. A similar reaction is not observed with 3,4-dihydroFAD released into solution upon denaturation of DAAO, suggesting that the reactivity of the 405 nm species bound to denatured MSOX may be affected by the covalent thioether link between the 3,4-dihydroflavin and the protein moiety. The absorption spectrum of the oxidized flavin obtained upon prolonged incubation of denatured MSOX might be modified by oxidation of the thioether link, but the spectral properties of the corresponding sulfone (λ_{max} = 350 and 450 nm) (*11*) are different from those observed for the modified flavin formed with MSOX. Similar spectral properties are observed for 4-iminoflavins at neutral pH (λ_{max} = 357−360 and 462−468 nm, pH 7.0), but these derivatives exhibit a pronounced bathochromic shift of the near-UV peak at acidic acid (λ_{max} = 388−392 and 462−470 nm, pH 2.0) (*12*), a feature that is not observed with the modified oxidized MSOX flavin.

The studies described in this paper were prompted by preliminary crystallographic analysis of borohydride-reduced CPG-modified MSOX which indicates that flavin C(4) is tetrahedral and a substituent has been introduced at flavin C(4a).[2] The presence of a tetrahedral carbon at flavin C(4) is consistent with the facile reduction of the C(4) carbonyl group observed in the current studies upon reaction of borohydride with unmodified MSOX. Interestingly, the substituent at C(4a) in borohydride-reduced CPG-modified flavin is likely to stabilize a hydroxy group at C(4) by preventing the tautomeric rearrangement reaction that appears to occur in its absence.

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