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Identification of a Stable Flavin-Thiolate Adduct in Heterotetrameric Sarcosine Oxidase

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Summary

Heterotetrameric sarcosine oxidase (TSOX) is a complex bifunctional flavoenzyme that contains two flavins. Most of the FMN in recombinant TSOX is present as a covalent adduct with an endogenous ligand. Enzyme denaturation disrupts the adduct, accompanied by release of a stoichiometric amount of sulfide. Enzyme containing ≥ 90% unmodified FMN is prepared by displacement of the endogenous ligand with sulfite, a less tightly bound competing ligand. Reaction of adduct-depleted TSOX with sodium sulfide produces a stable complex that resembled the endogenous TSOX adduct and known 4a-S-cysteinyl flavin adducts. The results provide definitive evidence for sulfide as the endogenous TSOX ligand and strongly suggest that the modified FMN is a 4a-sulfide adduct. A comparable reaction with sodium sulfide is not detected with other flavoprotein oxidases. A model of the postulated TSOX adduct suggests that it is stabilized by nearby residues that may be important in the electron transferase/oxidase function of the coenzyme.

Keywords

flavin mononucleotide; sarcosine oxidation; intramolecular electron transfer; oxygen reduction; thiolate adduct

Introduction

Heterotetrameric sarcosine oxidase (TSOX[¶]) is a complex bifunctional enzyme that catalyzes the oxidation of the methyl group in sarcosine (N-methylglycine) and the transfer of the oxidized methyl group into the 1-carbon metabolic pool. The enzyme contains four subunits (α, β, γ, δ), three coenzymes (NAD, FMN, FAD), a zinc ion and a binding site for tetrahydrofolate (H_4folate) [1–8]. Sarcosine binds near FAD where it is oxidized to the corresponding imine, accompanied by the formation of 1,5-dihydroFAD ($FADH_2$). Electrons from $FADH_2$ are transferred, one at a time, to FMN, which then reduces oxygen to hydrogen peroxide [9, 10]. In a second reaction, TSOX catalyzes the transfer of the oxidized 1-carbon

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Supplementary Data
Supplementary data associated with this article can be found, in the online version, at doi: xxxxxxxxxx.

¶Abbreviations: TSOX, heterotetrameric sarcosine oxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H_4folate, tetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; MSOX, monomeric sarcosine oxidase; MTOX, N-methyltryptophan oxidase; MMTS, methyl methanethiosulfonate; TCA, trichloroacetic acid; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; DAAO, D-amino acid oxidase; LAAO, L-amino acid oxidase; GOX, glucose oxidase.

fragment from the sarcosine imine ($CH_2=NH^+CH_2CO_2^-$) to H_4folate, forming 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) plus glycine [5, 6] (Supplementary Data, Figure S1). In the absence of H_4folate, turnover occurs at a somewhat slower rate and the sarcosine imine is hydrolyzed to glycine and formaldehyde. NAD does not participate in sarcosine oxidation[2] but appears to play an important structural role in TSOX [7].

TSOX is the most complex member of a family of amine oxidizing flavoenzymes. This family is mainly composed of considerably smaller, monomeric proteins (~44 kDa) that contain covalently bound FAD as the only prosthetic group and exhibit modest sequence homology (~20% identity) with the β subunit of TSOX. Members of this family include monomeric sarcosine oxidase (MSOX), N-methyltryptophan oxidase (MTOX), pipecolate oxidase and nikD [11–18]. Crystal structures have been determined for MSOX [11, 14], a monofunctional enzyme that exhibits sarcosine oxidase but not 5,10-CH_2-H_4folate synthase activity [5].

At least half of the covalent FMN in recombinant TSOX is present as a stable, but reversible, covalent complex with an unknown ligand; this complex exhibits negligible absorbance at 450 nm. The adduct is converted to unmodified FMN by reaction of the recombinant enzyme with methyl methanethiosulfonate (MMTS), sarcosine or hydrogen peroxide, a product formed during turnover with sarcosine. The spectral properties of the adduct and its reactivity with reagents known to oxidize (H_2O_2) or alkylate (MMTS) thiols suggested that the modified FMN might be a 4a-thiolate adduct [7, 19, 20]. Indeed, a 4a-S-cysteinyl flavin adduct is observable with certain forms of dihydrolipoamide dehydrogenase and mercuric reductase [21].

Expression of the recombinant TSOX in the presence of sarcosine yields adduct-free enzyme that could be crystallized, unlike adduct-containing enzyme [20]. The crystal structure, recently solved at 1.85 Å resolution [8], revealed that there was no nearby cysteine that might form a covalent adduct with FMN. In this paper we describe biochemical studies that show the FMN modification is due to the formation of a remarkably stable complex with a small molecule rather than an amino acid residue.

Effect of Sulfite Treatment on the Endogenous Adduct

The endogenous adduct in TSOX can be disrupted by MMTS or H_2O_2 but these reagents cannot be used to determine whether the adduct is formed by reaction of FMN with an amino acid residue or a small molecule that is not otherwise covalently attached to the protein. We sought to distinguish between these possibilities by dialysis of the enzyme in the presence of a reagent that disrupts the adduct without causing chemical modification of the ligand. We reasoned that a competing ligand might be an appropriate reagent since formation of the endogenous adduct is known to be reversible [7, 19]. Sulfite reacts selectively with FMN when present at a modest concentration (10 mM), forming a reversible covalent complex by nucleophilic addition at the N(5) position of the flavin ring [7]. This reagent was evaluated by conversion of TSOX to a FMN-sulfite adducted form, followed by dialysis against sulfite in order remove any displaced endogenous ligand. The sulfite adduct was then dissipated by dialysis against sulfite-free buffer. Untreated enzyme contained a substantial amount of endogenous adduct, as judged by the large increase in absorbance at 450 nm observed upon reaction with MMTS. In contrast, only a small spectral change was seen upon reaction of sulfite-treated enzyme with MMTS. The amount of adduct in untreated and sulfite-treated enzyme was estimated on the basis of the change in the spectral ratio, A_{280}/A_{450}, elicited by reaction with MMTS. Sulfite treatment decreased the adduct content from 69.0% to 10.4% of total FMN (Table 1). The results indicate that the ligand in the endogenous adduct is unlikely to be an amino acid residue.

Reaction of TSOX Extracts with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)

The sulfite dialysis experiment showed that the endogenous ligand is a small molecule that is probably attached to TSOX by a single reversible linkage involving FMN. We reasoned that the small molecule was likely to contain a sulfhydryl group, as judged by the spectral properties of the endogenous adduct and its reactivity with MMTS. Since enzyme denaturation disrupts the adduct, this treatment should release the small molecule into solution where it might be detected by reaction with a thiolate reagent like DTNB. To test this hypothesis, TSOX was denatured with 5% TCA, followed by centrifugation to remove the protein precipitate with its covalently attached FMN. The concentration of noncovalently bound FAD that had been released into the supernatant was determined based on the absorbance of the neutralized extract at 450 nm. Since TSOX contains equimolar amounts of noncovalently bound FAD and covalently bound FMN [19], the concentration of the putative thiolate ligand in the extract was estimated based on the concentration of FAD in the extract and the fraction of covalent FMN in intact enzyme that was present as the adduct.

A distinct odor of rotten eggs was detected upon denaturation of TSOX with TCA, suggesting that the thiolate ligand might be sulfide rather than an organic thiolate. Both organic thiolates and sulfide can be determined by reaction with DTNB based on the increase in absorbance at 412 nm due to formation of 2-nitro-5-thiobenzoate (TNB) (ε_{412} = 13,600 M^{-1} cm^{-1}). A 1:1 stoichiometry is observed with organic thiolates whereas the reaction with sulfide generates 2 mol of TNB [22, 23]. Formation of TNB was observed upon reaction of the TSOX extract with DTNB, as judged by the increase in absorbance at 412 nm (Table 2). The yield of TNB, as compared to the estimated thiolate content in the extract, was calculated by assuming either a 1:1 (66%) or a 2:1 (33%) reaction stoichiometry. The yield of TNB in control studies with sodium sulfide was 97% of the expected value when the assays were conducted with a freshly prepared sodium sulfide solution. However, the yield was reduced to 50% by prior treatment of sodium sulfide under the same conditions used to prepare the TCA extract with TSOX (Table 2). The decreased yield is probably due to loss as H_2S, as judged by the odor detected upon acidification. In contrast, cysteine was completely stable under the same conditions.

Sulfide Analysis

A spectrophotometric assay specific for sulfide was carried out by using a modification [24] of the method of Beinert [25]. In this assay, the protein is denatured by an overnight incubation with zinc hydroxide at room temperature and then mixed with reagents (N, N-dimethyl-*p*-phenylenediamine, $FeCl_3$) that generate an intense pink color in the presence of sulfide (ε_{670} = 34,500 M^{-1} cm^{-1}). Indeed, sulfide was detected in an untreated preparation of TSOX that contained 69.0% of its covalent FMN as the adduct. The amount of sulfide found was 80% of a value calculated based on the estimated adduct content (Table 3). A sample of the same preparation was also analyzed after dialysis against sulfite. This treatment caused a 7-fold decrease in the percentage of covalent FMN present as the adduct and was accompanied by a corresponding decrease in the sulfide content of the enzyme, as judged by the excellent agreement between observed and predicted values (Table 3). The results provide strong evidence in support of sulfide as the ligand in the endogenous adduct.

Reaction of TSOX with Sulfide

The known stability of the endogenous FMN adduct suggested that an identical complex could be generated by reaction of adduct-free TSOX with a small amount of sulfide. To test this hypothesis, the endogenous adduct was depleted by dialysis against sulfite to yield enzyme containing 8.4% residual adduct. An aliquot of this preparation (18.4 μM with respect to flavin) was then mixed with 20 μM sodium sulfide under anaerobic conditions. A nearly 40% loss of

flavin absorbance at 450 nm was observed in a reaction that was complete within 10 min. Only a small additional decrease could be detected when the sulfide concentration was doubled by addition of a second aliquot of the reagent. The absorption spectrum was virtually unchanged after exposure to air (Figure 1). The results show that reaction of TSOX with an approximately equimolar amount of sulfide results in the formation of an air-stable species that exhibits spectral properties similar to those observed for the endogenous adduct.

In a separate experiment, the enzyme was mixed with a 50-fold higher concentration of sodium sulfide. The spectrum recorded immediately after anaerobic addition of 1 mM sodium sulfide is similar to that observed for the reaction of TSOX with 20 μM sodium sulfide. A second slow phase was observed at the higher reagent concentration that resulted in considerable further bleaching of the flavin absorbance (Figure 1, inset). However, the spectrum obtained after aeration was similar to that recorded immediately after sulfide addition. The second phase of the reaction with 1 mM sodium sulfide is attributed to conversion of noncovalently bound FAD to 1,5-dihydroFAD, an air-sensitive species.

Reaction of Sulfide-treated TSOX with MMTS

The properties of the air-stable adduct formed upon treating TSOX with 20 μM sodium sulfide were further characterized by reaction with MMTS. Addition of MMTS resulted in a large, time-dependent increase in absorbance at 450 nm. The MMTS-treated sample exhibited spectral properties similar to that expected for adduct-free TSOX (Figure 2(a)). In contrast, only a small spectral change was observed in a control reaction with MMTS and an aliquot of the same enzyme preparation that had not been treated with sodium sulfide (Figure 2(a), inset). The observed disruption of the adduct with MMTS indicates that the reaction of TSOX with sulfide is reversible, although the equilibrium lies strongly in favor of complex formation, as observed with the endogenous adduct. The amount of adduct in the sulfide-treated enzyme preparation was estimated by comparing the spectral ratio, A_{280}/A_{450}, before and after treatment with MMTS. We found that 84.5% of the covalent flavin in sulfide-treated TSOX was present as a thiolate adduct, a 10-fold increase as compared with the corresponding untreated enzyme preparation (8.4 %). In a separate experiment, sulfide-treated TSOX was extensively dialyzed prior to reaction with MMTS. Dialysis caused only a small decrease in the percentage of covalent flavin present as an adduct (84.5 to 77.6%), providing further evidence regarding the stability of the adduct. The amount of sulfide found in the dialyzed sample was 91% of a value calculated based on the estimated adduct content (Table 3).

Calculated Absorption Spectrum of the Sulfide Adduct

The absorption spectrum observed after reaction of TSOX with sodium sulfide contains contributions due to the FMN-sulfide adduct plus unmodified flavin (residual uncomplexed FMN and noncovalently bound FAD). The percentage of FMN present as the sulfide adduct was determined based on the change in the spectral ratio, A_{280}/A_{450}, upon reaction with MMTS. The spectral properties of unmodified TSOX-bound flavin were estimated based on the absorption spectrum of the MMTS-treated sample. The absorption spectrum of the FMN-sulfide adduct was then calculated by subtracting the absorbance due to unmodified flavin. This approach assumes that the covalent FMN and noncovalent FAD exhibit identical spectral properties. This assumption is unavoidable, even though modest differences are likely, owing to difficulties encountered in attempts to prepare enzyme containing a single flavin [7]. The calculated spectrum of the sulfide-adduct (Figure 2(b)) exhibits a prominent absorption band at 386 nm ($\varepsilon_{386} = 7000\ M^{-1}\ cm^{-1}$); small negative and positive peaks at 469 and 493 nm, respectively, are artifacts, probably generated by differences in the spectral properties of the covalent and noncovalent flavins. The calculated spectrum of the sulfide-adduct closely resembles a spectrum calculated for the endogenous TSOX adduct ($\lambda_{max} = 383$ nm; $\varepsilon_{383} =$

7300 M^{-1} cm^{-1}) [19] and spectra reported for 4a-thiolate adducts in dihydrolipoamide dehydrogenase (λ_{max} = 380 nm; ε_{380} = 7000 M^{-1} cm^{-1}) and mercuric reductase (λ_{max} = 382 nm; ε_{382} = 7500 M^{-1} cm^{-1}) where the complexes are formed by reaction of the flavin with an active site cysteine [21].

Reaction of Other Flavoenzymes with Sulfide

Reversible nucleophilic addition of sulfite at the N(5) position of the flavin ring is a reaction characteristically observed with flavoprotein oxidases [26]. It was, therefore, of interest to determine whether formation of an adduct with sulfide was also a generic property of flavoprotein oxidases. We examined the anaerobic reaction of sulfide with several monomeric amine oxidases (MSOX, MTOX and nikD) that contain covalently bound flavin [8a-(S-cysteinyl)FAD] and exhibit modest sequence homology (~20% identity) with the β subunit of TSOX [11, 12, 18]. We also tested enzymes that contain noncovalently bound FAD: glucose oxidase (GOX) [27], yeast D-amino acid oxidase (DAAO) [28] and snake venom L-amino acid oxidase (LAAO) [29]. The reactions were conducted with 1 mM sodium sulfide since extremely slow reaction rates were observed with 20 μM sodium sulfide in preliminary studies with several of the enzymes. In all cases, incubation of the flavoprotein oxidases with 1 mM sodium sulfide resulted in enzyme reduction in reactions that proceeded via a flavin radical intermediate (Supplementary Data, Figure S2). A red anionic radical was observed with all enzymes, except for GOX where the intermediate consisted of a mixture of neutral and anionic radicals. The latter is consistent with the observed pH-dependence of the ionization state of the GOX radical, a feature that distinguishes this enzyme from most other flavoprotein oxidases [30]. The maximum radical yield (shown in parentheses) increased in the order: MTOX (56%), MSOX (66%), DAAO (75%), GOX (86%), LAAO (96%)[§]. In each case, conversion of the radical to 1,5-dihydroflavin occurred in a slower reaction. The fastest reactions with sodium sulfide were observed with LAAO, MSOX and MTOX where the radical reached a maximum concentration in less than 60 min and complete reduction occurred in less than 300 min. With nikD, glucose oxidase and DAAO, the maximum radical concentration was attained within about 200 to 400 min; conversion to fully reduced flavin was incomplete even after incubation for more than 20 h. In each case, aeration of the (partially) reduced enzymes restored the initial oxidized flavin spectrum. The results show that formation of a stable adduct with sulfide is an apparently unique feature of FMN bound to TSOX and not a general property of flavoprotein oxidases.

Concluding Remarks

Most of the covalently bound FMN in preparations of recombinant TSOX is found in a modified form, previously shown to involve formation of a reversible covalent adduct with an unknown endogenous ligand [7, 19]. The results obtained in this study provide definitive evidence for sulfide as the endogenous ligand in TSOX and strongly suggest that the modified FMN is a 4a-sulfide adduct. There is substantial kinetic evidence for the formation of 4a-thiolate adducts as intermediates in thiol-mediated flavin reduction reactions. However, the 4a-thiolate adducts are typically unstable and rarely detected owing to a rapid SN2 displacement of the sulfur by a second thiol anion in a reaction that generates 1,5-dihydroflavin [31, 32]. Indeed, the 4a-thiolate adducts in dihydrolipoamide dehydrogenase and mercuric reductase are observed only when the action of a second attacking thiol is blocked by alkylation or mutation [21]. Unlike TSOX, formation of a 4a-sulfide adduct is not observed with other flavoprotein oxidases, as judged by a survey of six enzymes where sulfide was found to act as an apparent 1-electron reductant, as reported for thioglycolate-mediated reduction of MSOX and MTOX [33, 34].

[§]The radical yield with nikD could not be calculated since the extinction coefficient of the radical is not known. The long wavelength absorption of oxidized nikD is due to an intrinsic charge transfer complex [17].

The results suggest that certain unique features of the FMN environment in TSOX might be responsible for the unusual stability observed for the 4a-sulfide adduct with this flavin. Consistent with this hypothesis, a model of the TSOX 4a-sulfide adduct indicates that the adduct is stabilized by hydrogen bonding between FMN N(5) and the carboxylate of β Glu280 as well as hydrogen bonding between the sulfur and the ε -amino group of β Lys278 (Figure 3(a)). (Steric factors may also contribute to adduct stability by blocking reaction with a second molecule of sulfide.) β Glu280 and β Lys278 are also ideally positioned to facilitate adduct formation (Figure 3(b)). Hydrogen sulfide anion (HS^-) is the predominant nucleophile in solutions of sodium sulfide at pH 8 (pK_a (H_2S) = 7.04, 11.96). Initial noncovalent binding of HS^- is probably driven by electrostatic interaction with the ε -amino group of β Lys278. Nucleophilic addition at flavin C(4a) is subject to general acid catalysis at flavin N(5) [31, 35]. The side chain of β Glu280 is hydrogen bonded to FMN N(5) in adduct-free TSOX [8] and likely to facilitate nucleophilic attack of HS^- at FMN C(4a) by acting as a general acid catalyst.

FMN in TSOX acts as an electron transferase/oxidase flavin, a catalytic role that differs from the dehydrogenase/oxidase functionality associated with the single flavin typically found in flavoprotein oxidases. Interestingly, the residues implicated in the unique reactivity of TSOX with sulfide are also likely to be important in catalysis. β Glu280 probably functions as a proton donor to FMN N(5) during interflavin electron transfer from FAD [8]. β Lys278 may play a role in oxygen activation by stabilizing the superoxide anion that is formed as an obligatory intermediate in oxygen reduction by reduced flavin [36]. Although chemically distinct, the 4a-thiolate adduct is structurally similar to a 4a-hydroperoxy adduct that has been proposed as a second intermediate in the reduction of oxygen.

In summary, we show that the covalently bound FMN in TSOX forms an unusually stable 4a-thiolate adduct with sulfide, probably by recruiting nearby residues that are likely to be important in the electron transferase/oxidase function of the coenzyme. Sulfide is produced in *E. coli* during assimilatory sulfate reduction for cysteine biosynthesis. Owing to its toxicity, the process is tightly regulated to prevent excess production of sulfide [37] but even trace amounts of sulfide are likely to be efficiently scavenged by the highly reactive FMN site in TSOX.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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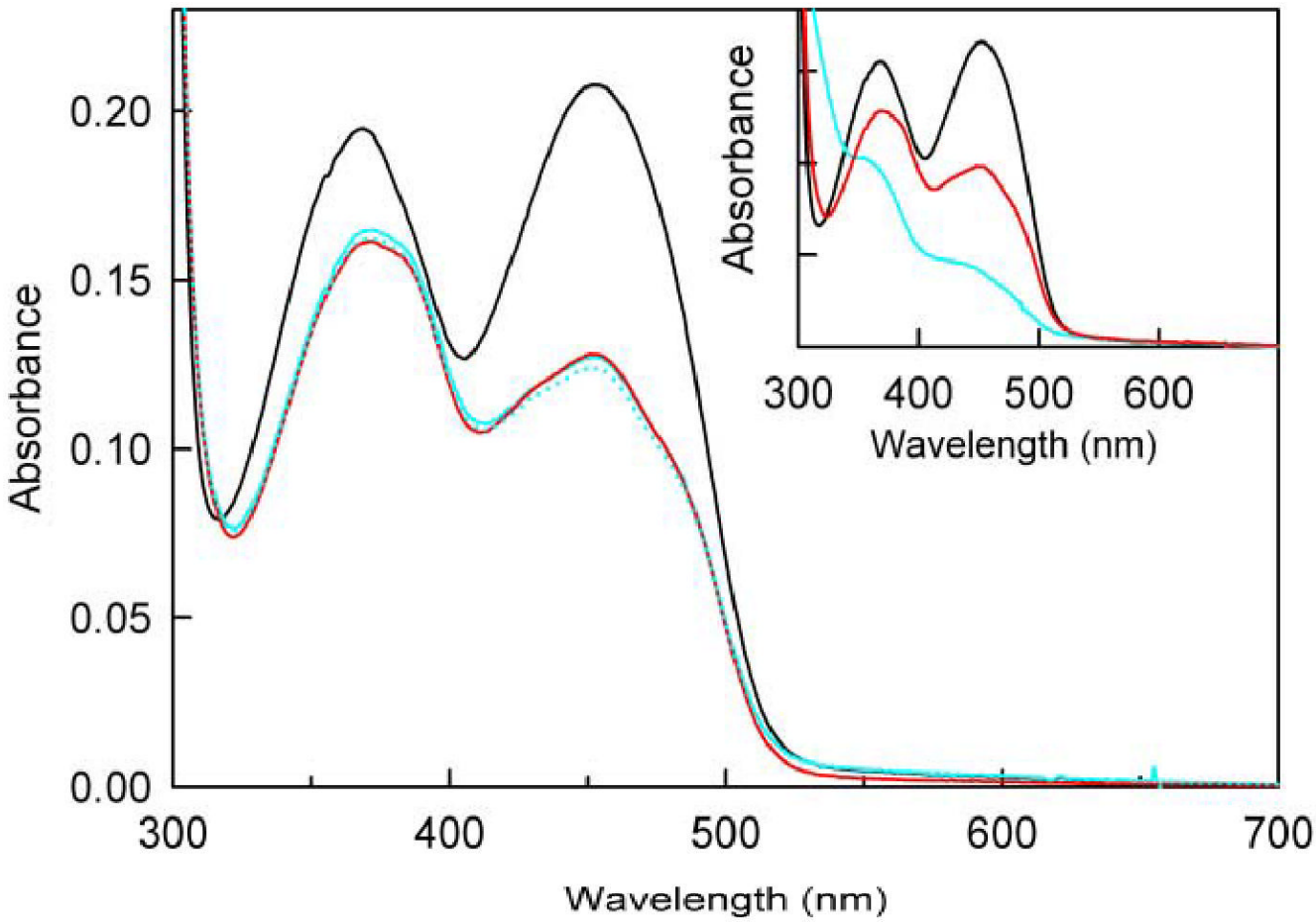

Figure 1.

Reaction of TSOX with sodium sulfide. The reaction was conducted under anaerobic conditions using enzyme that had been dialyzed against sulfite to deplete the endogenous adduct. The black curve is the initial absorption spectrum of TSOX (18.4 μM with respect to flavin) in 100 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA at 25 ° C. The solid cyan curve was recorded 10 min after addition of 20 μM sodium sulfide. The dotted cyan curve was recorded 45 min after a second addition that increased the sulfide concentration to 40 μM. The red curve was recorded 15 min after making the sample aerobic. The inset shows the reaction observed with TSOX and 1 mM sodium sulfide under the same conditions. The black curve is the initial spectrum. The red curve were recorded immediately after the addition of 1 mM sodium sulfide. The cyan curve was recorded after 105 min of reaction. The sample was then exposed to air. The spectrum recorded after aeration was similar to that observed immediately after sulfide addition (data not shown).

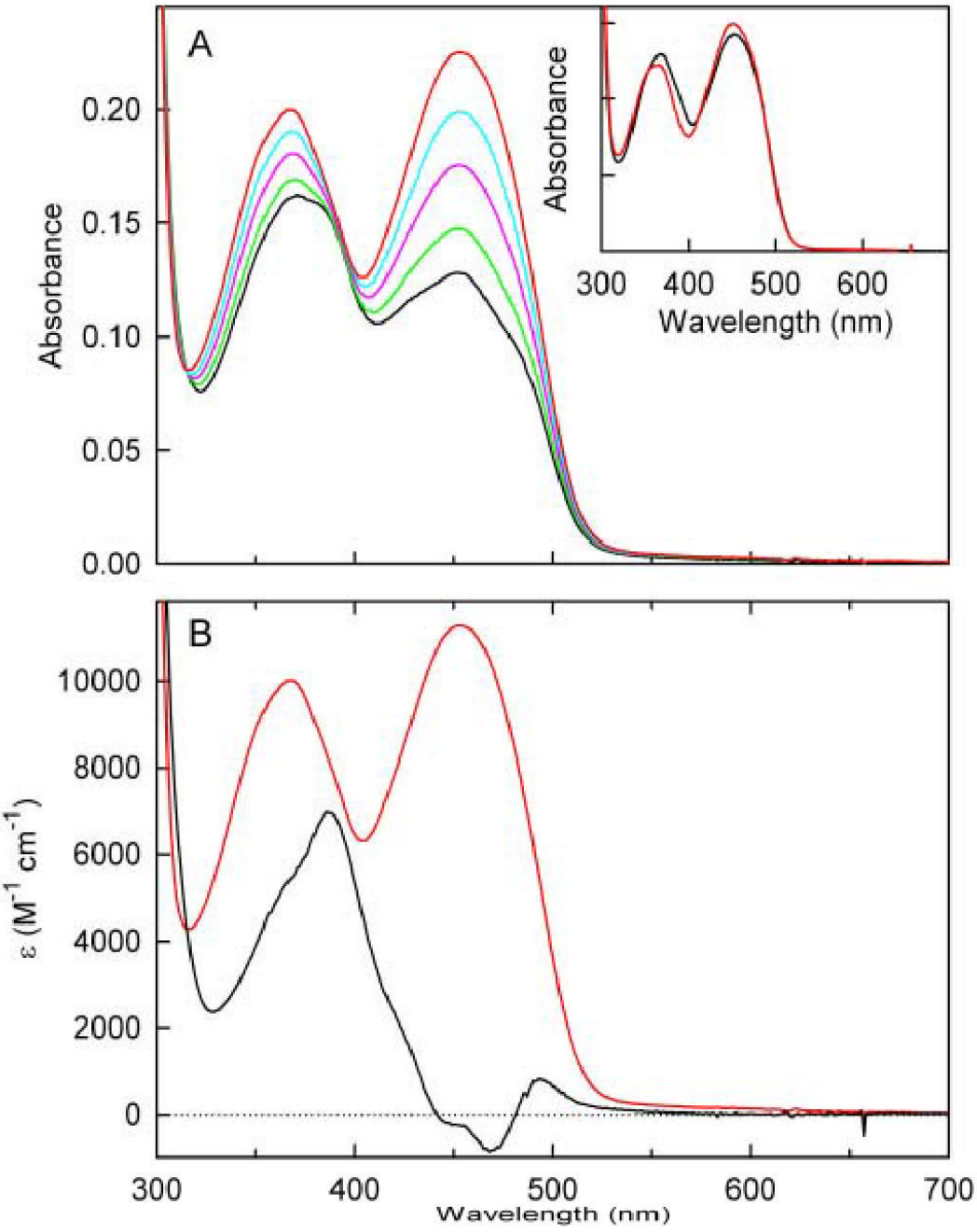

Figure 2.

Reaction of sodium sulfide-treated TSOX with MMTS (panel A) and calculation of the absorption spectrum of the adduct formed by reaction of TSOX with sodium sulfide (panel B). Panel A: The black curve is the absorption spectrum of sulfide-treated enzyme, prepared as described in the legend to Figure 1. The green, magenta, cyan and red curves were recorded 2, 5, 10 and 30 min, respectively after addition of 1 mM MMTS. The inset shows the reaction of MMTS with an aliquot of the same preparation that had not been treated with sulfide. Spectra recorded before and after reaction with MMTS are shown by the black and red curves, respectively. Panel B: The absorption spectrum of the sulfide adduct (black curve) was calculated by correcting the spectrum observed after reaction of TSOX with sodium sulfide

(Panel A, black curve) for the contribution due to unmodified flavin, as described in the text. For comparison, the red curve is the absorption spectrum of adduct-free TSOX that was obtained by reacting sulfide-treated enzyme with MMTS.

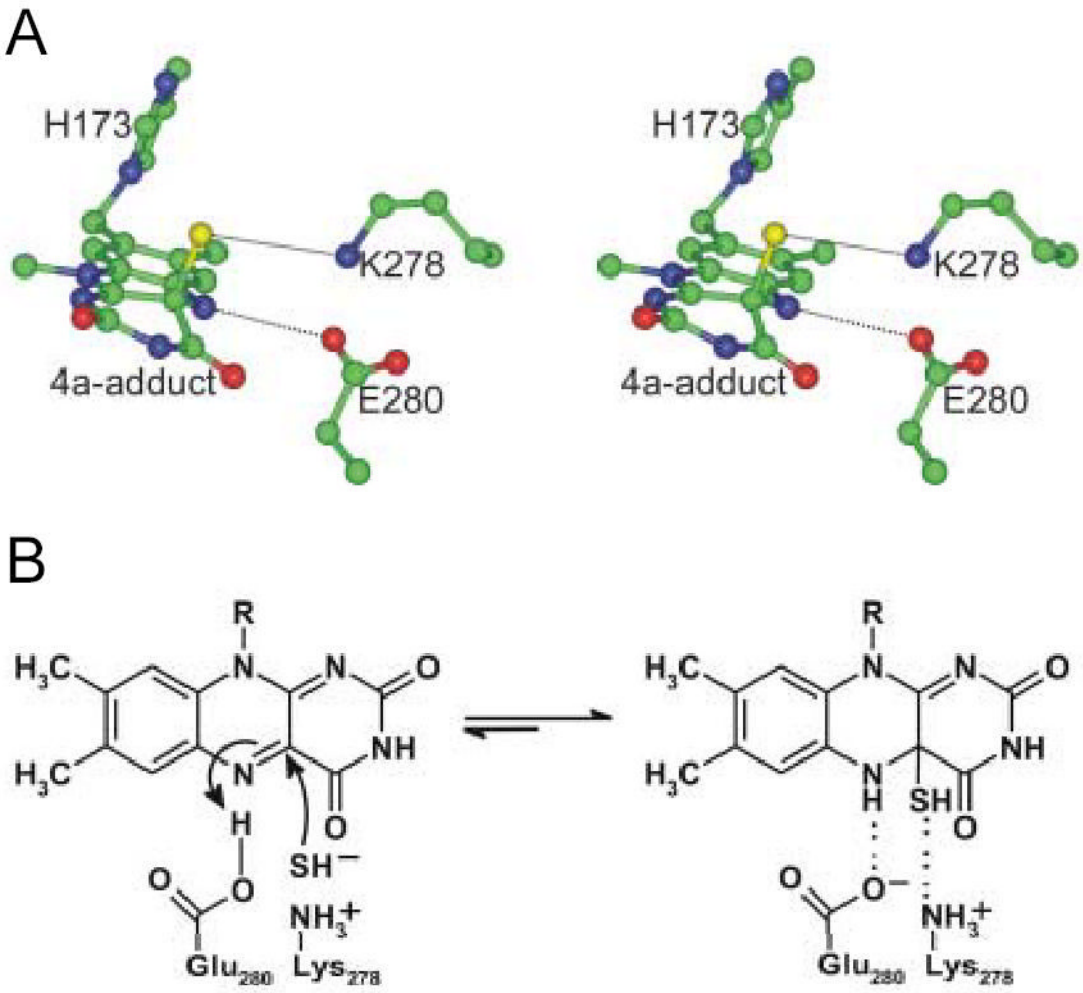

Figure 3.

Model of the 4a-thiolate FMN adduct (Panel A) and proposed mechanism for adduct formation during reaction of TSOX with sulfide (Panel B). Panel A: Stereoview of a model of the 4a-thiolate adduct of the covalent FMN and sulfide. Atoms are shown in atomic colors (C, green; O, red; N, blue; S, yellow). Hydrogen bonds between the adduct and Lys278 (3.59 Å) and Glu280 (3.28 Å), residues in the β subunit of TSOX, are indicated by dotted lines. The model was constructed based on the known structure of 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin [38] (Cambridge Structural Database entry HEHLFB10) using the molecular graphics package Turbo-Frodo [39]. The N(5)-attached atoms in the starting structure were discarded and replaced by a hydrogen. The oxygen bound to C(4a) was changed to sulfur and then the C(4a)-S4A bond was extended to a length of 1.8 Å. The model was then aligned with the unmodified FMN in the crystal structure of TSOX (PDB code 2GAG) [8]. Panel B: Initial noncovalent binding of HS^- is promoted by electrostatic interaction with β Lys278. Nucleophilic addition of HS^- at the C(4) position of the flavin ring is assisted by general acid catalysis by β Glu280 at N(5).

Table 1

Effect of sulfite dialysis on the spectral properties and endogenous 4a-adduct content of TSOX

	spectral properties[c]				
	before MMTS		after MMTS		
	A_{280}/A_{450}	A_{368}/A_{450}	A_{280}/A_{450}	A_{368}/A_{450}	4a-thiolate adduct (%)[d]
untreated[a]	18.0	1.13	11.8	0.81	69.0
sulfite dialysis[b]	12.9	0.88	12.2	0.83	10.4

[a] Recombinant TSOX from *Pseudomonas maltophilia* was purified and assayed as previously described [20] except that sarcosine was excluded from the growth medium.

[b] Enzyme depleted of the endogenous 4a-adduct was prepared by reaction with 10 mM sulfite in 20 mM potassium phosphate, pH 8.0, containing 1 mM EDTA, followed by dialysis for 24 h against buffer containing 10 mM sulfite and then dialysis for 24 h *versus* sulfite-free buffer.

[c] Absorption spectra were recorded before and 30 min after addition of 1mM MMTS in 10 mM potassium phosphate buffer, pH 8.0, at 25 ° C. Disruption of the 4a-thiolate adduct by MMTS results in a large increase in the ratio, A_{280}/A_{450}, and a smaller increase in the ratio, A_{368}/A_{450}. [Apparent extinction coefficients for adduct-containing preparations were estimated based on the increase in absorbance at 450 nm observed after reaction with MMTS, using an extinction coefficient previously determined with adduct-free TSOX (ε_{450} = 11,280 M^{-1} cm^{-1})].

[d] The 4a-thiolate adduct content was estimated by comparing values obtained for the ratio, A_{280}/A_{450}, before and after reaction with MMTS, as previously described [7].

Table 2

Reaction of DTNB with a TSOX extract or sodium sulfide[a]

	FAD (μ M)	predicted thiolate (μ M)	2-nitro-5- thiobenzoate (μ M)
TSOX extract[b]	29.2	20.2	13.5
sodium sulfide[c]			
untreated	-	40.0	77.8
TCA-treated	-	40.0	40.4

[a] Reactions with DTNB were conducted under aerobic conditions according to published methods [22]. Results are the average of duplicate runs.

[b] A sample of TSOX in standard buffer (20 mM potassium phosphate, pH 8.0, containing 1 mM EDTA) was denatured with 5% TCA and then centrifuged. The FAD concentration (ε_{450} = 11, 300 M^{-1} cm^{-1}) was determined based on the spectrum recorded for the neutralized supernatant. The predicted thiolate content was estimated as described in the text.

[c] A stock solution of sodium sulfide (prepared in anaerobic 0.002 N NaOH) was diluted into aerobic TSOX standard buffer. For the untreated sample, DTNB was added immediately. For the TCA-treated sample, the dilution in TSOX buffer was acidified with TCA, incubated on ice for 45 min, neutralized and then reacted with DTNB.

Table 3

Analysis for the presence of sulfide in TSOX preparations[a]

	covalent FMN (μ M)	4a-thiolate adduct (%)	sulfide (μ M)	
			predicted[b]	observed
untreated	15.9	69.0	11.0	8.72
sulfite-dialyzed				
0 μ M Na_2S	15.0	10.4	1.56	1.56
20 μ M Na_2S[c]	7.07	77.6	5.48	4.99

[a] Sulfide analyses were conducted according to published methods [24, 25]. Results are the average of duplicate runs.

[b] The predicted concentration of sulfide was calculated based on the total amount of covalent FMN added to the assay and the fraction of covalent FMN in the enzyme preparation that was present as the adduct, as judged by the reaction with MMTS.

[c] The Na_2S-treated sample was dialyzed prior to sulfide analysis.