Structure and function of a flavin-dependent S-monoxygenase from garlic

(*Allium sativum*)

Hannah Valentino¹,², Ashley C. Campbell³, Jonathan P. Schuermann⁴, Nazneen Sultana¹, Han G. Nam¹, Sophie LeBlanc¹, John J. Tanner³,⁵,* and Pablo Sobrado¹,²,*

¹Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061
²Center for Drug Discovery, Virginia Tech, Blacksburg, VA 24061
³Department of Biochemistry University of Missouri, Columbia, MO 65211
⁴Northeastern Collaborative Access Team, Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853
⁵Department of Chemistry, University of Missouri, Columbia, MO 65211

*Corresponding authors:
psobrado@vt.edu and tannerjj@missouri.edu

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Abstract

Allicin is a component of the characteristic smell and flavor of garlic (*Allium sativum*). A flavin-containing monoxygenase (FMO) produced by *A. sativum* (AsFMO) was previously proposed to oxidize S-allyl-L-cysteine (SAC) to alliin, an allicin precursor. Here, we present a kinetic and structural characterization of AsFMO that suggests a possible contradiction to this proposal. Results of steady-state kinetic analyses revealed that AsFMO exhibits negligible activity with SAC; however, the enzyme was highly active with L-cysteine, N-acetyl-L-cysteine, and allyl mercaptan. We found that allyl mercaptan with NADPH is the preferred substrate–cofactor combination. Rapid-reaction kinetic analyses showed that NADPH binds tightly (*K_D* ~2 μM) to AsFMO and that the hydride transfer occurs with pro-R stereospecificity. We detected formation of a long-wavelength band when AsFMO was reduced by NADPH, probably representing the formation of a charge transfer complex. In the absence of substrate, the reduced enzyme, in complex with NADP⁺, reacted with oxygen and formed an intermediate with a spectrum characteristic of C4a-hydroperoxyflavin, which decays several orders of magnitude slower than the *k_cat*. The presence of substrate enhanced C4a-hydroperoxyflavin formation, and upon hydroxylation, oxidation occurred at a rate constant similar to the *k_cat*. The structure of AsFMO complexed with FAD at 2.08 Å resolution features two domains for binding of FAD and NADPH, representative of class B flavin monoxygenases. These biochemical and structural results are consistent with AsFMO being an S-monoxygenase involved in allicin biosynthesis by direct formation of sulfenic acid, and not by SAC oxidation.

Introduction

Garlic (*Allium sativum*) is one of the best known and most consumed spices worldwide, serving as a key ingredient in many dishes. In addition to being used for its flavor and aroma, garlic has also been utilized as a nutraceutical for thousands of years. Records, dating as far back as 2700 BC in Asia, indicate that garlic was used in remedies for treating skin infections, gastrointestinal diseases, bronchitis, and cardiovascular illness (1). Garlic’s distinct taste is due, in part, to the production of organosulfur compounds, specifically allicin (S-allyl prop-2-ene-1-
sulfinothioate) and its allyl sulfide derivatives, such as diallyl disulfide, diallyl trisulfide, and ajoene. Extensive experimentation has shown that these compounds exhibit numerous health properties, including antimicrobial, anticancer, anti-inflammatory, and cardiovascular benefits (2-8). Agricultural benefits from allicin have also been described, showing potential application in organic farming (9). One of the main issues with using these compounds is that they are very reactive and organosulfur production varies greatly between garlic plants, depending on soil, temperature, season, and other factors, making standardization of allicin production through agricultural means a challenging task (10-12). Therefore, a better understanding of the enzymes involved in this pathway would make it possible to engineer allicin biosynthesis for flavor and medicinal applications.

In garlic, allicin plays a role in the defense mechanism that is initiated after damage to the plant tissue. Radiolabeling studies have led to the proposal that there are two allicin biosynthetic pathways, starting with either L-serine or L-glutathione (5,13,14). Both pathways involve the incorporation of an allyl donor from an unknown source, which, ultimately, leads to the synthesis of the stable sulfoxide alliin (Scheme 1). In response to plant injury, alliin is hydrolyzed by alliinase into allyl sulfenic acid and pyruvate with the consecutive nonenzymatic reaction of two allyl sulfenic acids producing allicin. A key enzyme involved in allicin biosynthesis is a flavin-containing monooxygenase (FMO), which has been proposed to convert S-allyl-L-cysteine (SAC) into alliin through a chiral sulfoxidation reaction (Scheme 1) (15).

FMOs belong to the large family of flavin-dependent monooxygenases, specifically to the subclass B monooxygenases, which are important players in many biosynthetic pathways due to their ability to oxygenate a range of substrates, including aromatic carbons, ketones, and soft nucleophilic heteroatoms (16,17). The general reaction of members of this class is divided into reductive and oxidative half-reactions. The reductive half-reaction involves binding of NAD(P)H, hydride transfer to FAD, and formation of the reduced enzyme in complex with NADP+. Retention of NADP+ by the reduced enzyme is important for the oxidative half-reaction, as it is essential for the formation and stabilization of the C4a-hydroxyflavin species, which is the intermediate that performs the hydroxylation of the aromatic, N- and S-containing compounds. (18-20). These reactions are highly stereospecific, which makes FMOs attractive targets for industrial applications (21). FMOs from plants contain the largest number of enzymes and have been shown to be involved in cell signaling, defense, and detoxification (22). FMOs from this kingdom are separated into three clades according to sequence homology. Clade I FMOs are involved in plant defense, as represented by the recently characterized enzyme from Arabidopsis thaliana, which catalyzes N-hydroxylation of pipoelic acid (23). Clade II includes YUCCA enzymes, which are involved in auxin biosynthesis (24). Clade III can be best described as FMO GS-OX1-5, which is involved in the S-oxidation of the anticarcinogenic compounds, glucosinolates (GSLs) (25). Previous work by Saito and colleagues suggested that the FMO from AsFMO, as well as similar FMOs from other Allium plants, belong to Clade III and are involved in the biosynthesis of S-alk(en)yl-L-cysteine sulfoxides, including alliin and similar sulfur compounds, methiin, propin, and ethiin (14). While there have been some reports of AsFMO activity in yeast cell extracts as well as in vivo activity of similar enzymes (15, 26-28), the isolation and in vitro characterization of this enzyme has yet to be accomplished. In this work, we present the biochemical and structural characterization of AsFMO, as well as a detailed understanding of the function of this enzyme, providing biochemical evidence of its role in the biosynthetic pathway for allicin production in plants.

**Results**

**Protein expression and purification**

Recombinant AsFMO was initially expressed as an N-terminus 6xHis-fusion protein; however, this form of the protein was highly insoluble. To overcome this problem, the protein was then expressed as a fusion with 6xHis-maltose binding
protein (MBP) and purified using immobilized metal affinity chromatography (IMAC). The MBP-AsFMO fusion protein was treated with tobacco etch virus (TEV) protease and the free AsFMO was stable and could be isolated to >95% homogeneity, as determined by SDS-PAGE (Fig. S1). The protein yield was 1.3 ± 0.2 mg protein per 1 g of cell pellet. It was determined by mass spectrometry that the protein contained FAD (data not shown) and around 50% of the protein contain flavin. The absorbance spectrum of bound flavin was very similar to free FAD (Fig. S2). The extinction coefficient of FAD bound to AsFMO at pH 7.5 was determined to be 12.8 mM⁻¹ cm⁻¹ at 450 nm.

**Steady-state kinetics**

The activity under steady-state conditions was monitored following oxygen consumption. AsFMO showed an initial velocity of 0.06 ± 0.005 s⁻¹ when SAC was used as the substrate, which resembled the background activity with no substrate (Fig. 1A). These results were also obtained when the buffer conditions were changed to sodium phosphate pH 7.5 or Tris-Cl pH 8.0. Even when a reaction of 0.5 µM enzyme, 15 mM SAC and 5 mM NADPH was incubated for 5 hours, there were still no observable changes in SAC concentration (Fig. S3A).

To identify potential substrates for AsFMO, the allin biosynthetic pathway as well as other thiol containing compounds were reviewed. S-methyl-L-cysteine (SMS), glutathione (GSH), L-cysteine (Cys), N-acetyl-L-cysteine (NAC), allyl mercaptan (AM), thiopropane, thioethane, and thiomethoxide were selected and tested as potential substrates. From these, Cys, NAC, and AM exhibited significant activity (Fig. 1A), where the others showed negligible or no activity. We also monitored the levels of Cys and showed that it decreases as a function of time (Fig. S3B). The kinetic parameters obtained from the oxygen consumption assays are summarized in Table 1. The results show a 2-3-fold higher $k_{cat}$ value with Cys compared to AM and NAC; however, the $K_M$ value is 5-10-fold lower for AM resulting in a 2-9-fold higher $k_{cat}/K_M$ value. AsFMO exhibited a 2-7-fold higher $k_{cat}/K_M$ value with NADPH compared to NADH originating from a lower $K_M$ value for NADPH. Substrate inhibition was observed only with excess NADPH with a $K_I$ value of 4 mM (Fig. 2B).

**Product formation with Cys**

The product of the enzyme reaction with Cys was determined by mass spectrometry (Fig. S4). Cys was identified in the reaction mixture with an m/z value of 122.027, which matches the values observed for Cys in the control samples. A peak at 241.033 m/z was observed in the reaction mixture, consistent with formation of L-cystine. Control experiments with an L-cystine standard supported this assignment. The peak was only present in the reaction mix and not observed in the non-enzyme control (Fig. S4). From these observations, it is likely that AsFMO hydroxylates the thiol group on Cys forming Cys sulfenic acid. The product then reacts nonenzymatically with excess Cys to form L-cystine (Scheme S1). Sulfenic acid detection was attempted using the derivatization agents 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and cis-5-norbornene-endol,3-dicarboxylic acid with no success (data not shown). It is possible that the reactivity of Cys sulfenic acid with excess free Cys prevented derivatization.

**Hydrogen peroxide formation**

While FMOs commonly perform hydroxilation reactions, they are also known to produce hydrogen peroxide as part of an unproductive or uncoupled reaction (29-32). This usually occurs very slowly when there is no substrate, or when an inhibitor or non-optimal substrate binds to the active site. If $H_2O_2$ is produced, it is possible that it will nonenzymatically react with Cys, producing Cys sulfenic acid and, subsequently, L-cystine (33). Therefore, determining the rate of the uncoupled reaction was essential in determining whether the observed activity was enzymatically produced or was a byproduct of uncoupling. This was accomplished by comparing the change in the rate of oxygen consumption of AsFMO in the presence of catalase. Catalase was selected for this experiment as it converts $H_2O_2$ into water and oxygen that can be directly measured as a decrease in the initial rate of oxygen consumption.
By monitoring NADPH oxidation, it was determined that 1 mg/mL catalase did not inhibit the enzyme activity; however, there was a decrease in the activity measured by monitoring oxygen consumption (Fig. S5). The percentage of the enzymatic reaction of Cys, NAC, and AM that was uncoupled with NAD(P)H under saturated conditions (Fig. 2) was much lower than that which was with NADH, suggesting that NADPH is the preferred cofactor. The reaction with NADPH and AM was the least uncoupled at ~1%, compared to 23% with Cys and 35% with NAC.

**Determination of the oligomeric state**

Size exclusion chromatography was used to determine that recombinant AsFMO exists in two oligomeric states; 151,000 ± 4,000 Da and 57,000 ± 2,000 Da, which correlate to trimeric and monomeric forms, respectively (predicted mass based on amino acid composition is 51,700 Da) (Fig. S6, Table S1). These two forms were observed using different enzyme preparations, with 12 ± 2% being trimeric and the rest being monomeric. When the enzyme was incubated with Cys prior to separation based on size exclusion, the trimeric form was no longer observed. When the same experiment was repeated with NADP+, no changes were noticed (data not shown).

**Reductive half-reaction**

The reaction of oxidized AsFMO with NAD(P)H was monitored using a stopped flow spectrophotometer under anaerobic conditions. With NADPH in the absence of Cys, a bleaching of the peak at 450 nm occurred in two phases. In addition, we observed that a stable broad band formed between 520-650 nm (Fig. 3) that correlated to the slow phase of reduction at 450 nm. The fast phase ($k_{\text{fast}}$) corresponded to 82% of the amplitude change. The rate constants of the slow phase ($k_{\text{slow}}$) and the formation of the broad band ($k_{570}$) were much slower than the $k_{\text{cat}}$ with Cys (Table 2). The value for $k_{\text{fast}}$ was constant even at 20 μM NADPH; thus, we estimate that the dissociation constant ($K_d$) for NADPH for this enzyme population is much lower (at least 10 times lower). The $k_{\text{slow}}$ was dependent on NADPH concentration and a $K_D$ of 15 μM was determined for this enzyme form. Formation of the long wavelength band also exhibited dependence on NADPH concentration with a $K_D$ of 33 μM. When AsFMO was incubated with Cys prior to mixing with NADPH, reduction occurred as a single phase, and the formation of the long wavelength band was only observable at low concentrations of NADPH. When AsFMO was reduced with NADH without Cys, the reaction was also biphasic, however, the formation of the long wavelength band was not observed (Fig. S7). The value for both rate constants were slower than observed with NADPH. In addition, the $K_D$ for NADH was much higher than for NADPH (Table 2).

The stereo-specificity of hydride transfer was probed using deuterated pro-R-4-2H-NADPH or pro-S-4-2H-NAPDH to reduce AsFMO when no substrate was present. From this experiment, it was observed that AsFMO exhibits a kinetic isotope effect (KIE) of 5.4 ± 0.1 with pro-R-4-2H-NADPH and 2.2 ± 0.05 with pro-S-4-2H-NADPH, demonstrating stereo-specificity for pro-R hydrogen. Formation of the long wavelength band ($k_{570}$), as well as the second phase of reduction ($k_{\text{slow}}$), are not affected by isotopic substitution (Fig. S8, Table S2).

**Oxidative half-reaction**

During the oxidative half-reaction, a peak rapidly formed at 370 nm, consistent with formation of the C4a-hydroperoxyflavin intermediate (Fig. 4). This peak is most prominent in the absence of substrate, decaying after ~ 7s into the fully oxidized enzyme. The rate of C4a-hydroperoxyflavin intermediate formation ($k_{\text{OOH}}$) was enhanced in the presence of Cys. The rate of oxidation in the absence of Cys ($k_{H_2O_2}$), which reports on H$_2$O$_2$ elimination, was several orders of magnitude slower than $k_{\text{OOH}}$. In the presence of Cys, turnover occurs, and the observed change in absorbance corresponds to substrate hydroxylation ($k_{\text{ox}}$), (Table 3). This process occurs in two phases, with the $k_{\text{ox,slow}}$ having a value similar to $k_{\text{cat}}$ with Cys. Decay of the long wavelength band from 520-650 nm was ~1-2 s$^{-1}$, with and without Cys.
Depending on the protein batch the oxidation of AsFMO varied. Some preparations exhibited a two-phased oxidation without Cys instead of a single phase oxidation (Fig. S9). The fast phase of this experiment was $3.3 \pm 0.3$ s$^{-1}$, which was very similar to the $k_{\text{ox,fast}}$ observed during oxidation with Cys. Both rates are much faster than the $k_{\text{cat}}$ value.

Structure determination

The structure of AsFMO complexed with FAD was determined at 2.08 Å resolution using Se-Met single-wavelength anomalous diffraction phasing (Fig. 5A). The structure exhibits the expected fold for an FMO, consisting of large and small, 3-layer $\beta$-\$\alpha$ domains. The larger domain binds FAD, while the smaller one presumably binds NADPH. The FAD binds with its ADP moiety mainly interacting with the larger domain, and the isoalloxazine located in the space between the two domains (Fig. 5A). The $si$ face of the isoalloxazine is buried, while the $re$ face is available for catalysis. This is expected for FMOs. Analysis of the protein-protein interfaces in the crystal lattice with PDBePISA(34) revealed no stable interfaces, suggesting AsFMO may be predominantly monomeric in solution.

Query of the PDB with PDBeFold (35) revealed several FMOs as structural neighbors of AsFMO (36-40), the closest being pyrrolizidine alkaloid N-oxygenase from the grasshopper Zonocerus variegatus (ZvPNO, PDB ID 5NMW, 31% sequence identity to AsFMO) (38). AsFMO and ZvPNO superimpose with an RMSD of 1.4 Å (Fig. 5B). The C-terminus is a point of departure between the two structures; AsFMO has a 13-residue $\alpha$-helix at the C-terminus, whereas ZvPNO has a $\beta$-hairpin. The FADs of AsFMO and ZvPNO have slightly different ribityl conformations (Fig. 5C). In AsFMO, the 3’-OH is below the pyrimidine ring of the isoalloxazine and is hydrogen bonded to the FAD N1. In ZvPNO, the 3’-OH points toward Tyr 307. The difference may be due to the replacement of Tyr307 with Pro342 in AsFMO. Steric clash with Pro342 would prevent the 3’-OH in AsFMO from adopting the conformation seen in ZnPNO. As a result, the two flavins differ by a crankshaft rotation involving the 2’-OH and 3’-OH. We note that the FAD conformation of AsFMO is very similar to that of FMO from Schizosaccharomyces pombe (PDB ID 2GV8), which also has a Pro342 (40).

Discussion

AsFMO has been reported to perform a chiral oxidation on SAC forming alliin, the stable precursor of allicin (15). This proposed function has been generally accepted in the literature, with a prior publication reporting that recombinant AsFMO in yeast extracts exhibits activity with SAC, albeit at a low rate ($V_{max}= 3.4 \pm 1.2$ pmol µg$^{-1}$ h$^{-1}$) (14,15,22, 41, 42). One of the aims of this study was to characterize the proposed enzymatic activity and further validate the role of AsFMO in allicin biosynthesis. However, despite extensive attempts, we were unable to demonstrate activity of AsFMO with SAC. This lack of activity was not due to isolation of an inactive enzyme, since we tested several other potential substrates and showed that the enzyme was active with Cys, NAC, and AM. Furthermore, with AM, AsFMO was highly coupled. Analysis of the catalytic efficiencies ($k_{\text{cat}}/K_M$) and coupling, suggest that AM and NADPH are the preferred substrates (Table 1). Clearly, our data does not support the proposed activity of AsFMO with SAC, but instead suggests that AsFMO contributes directly to the biosynthesis of organosulfur compounds by oxidizing AM into allyl sulfenic acid (Scheme 2). This physiological role is supported by the fact that AM is a significant garlic metabolite making up 1.4% of the volatile compounds in garlic (43,44). In addition, AsFMO would be regulated by the restrictive oxygen conditions inside the garlic cytosol, fitting with the current assumption that plant tissue damage is essential for allicin production, as AsFMO will be active when exposed to atmospheric oxygen (12, 45).

The observed activity with Cys and NAC could also be of physiological relevance, as Cys has been shown to be important for biosynthesis of organosulfur compounds in garlic (46-48). NAC has also been reported as abundant in water-soluble extracts of garlic samples and it is also believed to be in the biosynthesis of N-acetyl-S-allyl-L-cysteine (49, 50). The activity of AsFMO with Cys could account for production of L-
cystine in garlic (0.65 mg/g according to the USDA-Food Data Central ID: 169230). The specific role of cystine in garlic or other Allium plants is unknown; however, it has been shown that alliinase from A. sativum exhibits cystine lyase activity, which, when performed in the presence of potassium polysulfide, can lead to the formation of organosulfur compounds like diallyl disulfide and diallyl trisulfide (51).

Stopped-flow kinetic analysis allowed for the detailed characterization of the catalytic cycle of AsFMO (Scheme 3). The reductive half-reaction starts with the oxidized enzyme binding NADPH with high affinity (Scheme 3A and B). Flavin reduction occurs in two phases with pro-R stereospecificity (Scheme 3C). The fast phase is much faster than $k_{cat}$, indicating that it is not the rate-limiting step of the reaction. We suggest that the slow phase, which consists of only 18% of the amplitude change, represents the reaction of an enzyme population that is present in a different conformation (see below). Substrate binding can occur before or after NADPH binding, and only slightly decreases the rate constant for flavin reduction. This effect has been observed in other class B FMOs and is thought to be caused by conformational changes induced by substrate binding (31). The oxidative half-reaction starts with reaction of oxygen with the reduced enzyme in complex with NADP$^+$ (Scheme 3D). The C4a-hydroperoxyflavin can be observed with a characteristic peak at 370 nm (Fig. 4A). In the absence of hydroxylatable substrate, this intermediate is stable, and the enzyme oxidizes by the slow release of H$_2$O$_2$ (~300 times slower than $k_{cat}$) (Scheme 3D). This is due to stabilization by NADP$, which is essential to ensure coupling of the reductive and oxidative half-reactions. In the presence of hydroxylatable substrate, formation of the C4a-hydroperoxyflavin is slightly enhanced. Here, hydroxylation and flavin oxidation take place by release of H$_2$O and other products (Scheme 3E and F). This process occurs in two phases and the $k_{oxslow}$ and $k_{cat}$ values are very similar, suggesting that flavin oxidation is at least partially rate-limiting (Table 3). We noticed that the amplitude of the $k_{oxfast}$ is not the prominent phase (~30%) and varies between experiments. It is possible that this observed fast phase is related to a population of enzyme that does not stabilize the C4a-hydroperoxyflavin intermediate. Interestingly, the amplitude of the fast phase is similar to the percentage of uncoupling observed at saturating conditions of Cys and NADPH (23%; Fig. 2). This suggests that the population of enzyme contributing to the fast phase of oxidation could be responsible for the uncoupled reaction.

The kinetic characterization is consistent with AsFMO being a member the class B flavin-dependent monooxygenase mechanism (52). Members of this class share specificity for NADPH, pro-R stereoselectivity, and stabilization of the C4a-hydroperoxyflavin, as demonstrated for AsFMO (29, 53-55).

A unique aspect observed with AsFMO was the formation of a broad band between 520-650 nm when reduced by NADPH. This band is very stable, only decaying when the reduced enzyme is exposed to molecular oxygen. It is possible that this band is the formation of a charge-transfer complex. This assumption is reasonable as charge-transfer complexes have been reported in other FMOs, including the plant enzyme YUCCA monooxygenase (56). The fact that AsFMO reduction at 450 nm is completed in two phases, with the slow phase correlated to the formation of the long wavelength band, suggests that there are two populations of AsFMO that interact differently with NADP$. The slow phase at 450 nm is not isotope-sensitive, suggesting that either the change in absorbance is related to conformational changes or that, with this enzyme population, hydride transfer is not rate limiting. The possibility of different enzyme forms is supported by the size-exclusion chromatography results that show the presence of a trimeric form that is absent in the presence of Cys. The slow rate associated with the long wavelength band formation (0.03s$^{-1}$), especially when compared to the $k_{cat}$ under similar conditions (1.2 s$^{-1}$), demonstrates that it is likely not catalytically relevant. It is possible that this enzyme population is the one reacting faster with oxygen ($k_{oxfast}$), leading to uncoupling.

The structure of AsFMO complexed with FAD was determined at 2.08 Å. This structure contains a two-domain architecture for binding of FAD and NADPH, common in Class B flavin
monooxygenases. The active site shows that the re face of the isoalloxazine ring is exposed for catalysis. There are structural homologs of AsFMO from Zonocerus variegatus, which is proposed to be a pyrrolizidine alkaloid N-oxygenase. There are some differences in the interaction with the flavin cofactor, which are also observed in the FMO from S. pombe.

The structure provides insight into the preference for AM over SAC as the substrate. A reasonable model of AsFMO complexed with NADP⁺ was built by supimposing the structure of S. pombe FMO complexed with NADPH (PDB code 2GV8) onto the AsFMO structure. Inspection of the model revealed a tunnel that provides access to the C4a of the FAD, which we propose to be the substate-binding tunnel. Steric constraints suggest that the substrate must be threaded through a narrow entranceway (Fig. 6A). A model of SAC in the tunnel suggests there may not be sufficient room to accommodate the three terminal C atoms of SAC while bringing the S atom close to the C4a (Fig. 6B). No such impediment is observed with AM (Fig. 6C).

In conclusion, this paper describes the activity and kinetic mechanism of AsFMO. Unexpectedly, AsFMO does not exhibit significant activity with SAC, the generally accepted in vivo substrate. Instead it is active with Cys, NAC, and AM, with a distinct preference for AM. Thus, our data does not support the generally accepted role of AsFMO in allicin biosynthesis (Scheme 1). We propose that AsFMO catalyzes a feeder pathway for allicin biosynthesis by catalyzing the oxidation of allyl mercaptan to allyl sulfenic acid (Scheme 2). Presumably, an as-yet undiscovered enzyme catalyzes the oxidation of SAC to allinin. The crystal structure and biochemical characterization represent the first for specific flavin S-monooxygenase from plants.

**Experimental procedures**

**Materials**

The gene coding for AsFMO (Accession number: AB924383) was synthesized and the codon-optimized by GenScript (Piscataway, NJ). It was subcloned into pET15b for expression with an N-terminal 6xHis tag in Escherichia coli. Primers for subcloning and sequencing were purchased from Integrated DNA Technologies (Newark, NJ). Oneshot™ BL21 (DE3) and Top10 E. coli cells were purchased from Thermo Fisher Scientific (Waltham, MA) and used for protein expression and DNA amplification, respectively. Reagents for AsFMO crystallization were from Hampton Research (Aliso Viejo, CA). All gases were purchased from Airgas (Radnor, PA). Stereo-specifically deuterated, reduced nicotinamide phosphate (pro-R-4-2H-NADPH and pro-S-4-2H-NADPH) were synthesized following published procedures (57).

**Gene subcloning and protein expression**

The pET15b vector expressed a protein that was largely insoluble. To overcome this problem, the AsFMO gene was inserted into the pVP56K vector in frame for expression with an N-terminal 8xHis-MBP, as described previously (58-60). Protein expression was performed in BL21(DE3) cells using an autoinduction method, as previously described (60). Cells were harvested by centrifugation at 5,000 g and stored at -70 °C. For expression of selenomethionine (Se-Met)-labelled AsFMO (SeMet-AsFMO), a minimal media kit, purchased from Medicillin (Chicago, IL.), was used. Cells were grown in Luria-Bertani (LB) broth for 8 hours at 37 °C with agitation at 250 rpm. 50 mL of minimal media was inoculated with 1 mL of the LB culture and grown overnight at 37 °C, with agitation at 250 rpm. Protein expression was performed in 1 L minimal media inoculated with 10 mL of the overnight culture. The culture was grown at 37 °C with constant agitation (250 rpm) until an OD₆₀₀ of 0.6 was reached. To inhibit methionine production, 0.15 g of leucine, isoleucine, and valine, and 0.3 g of lysine, threonine, and phenylalanine were added, along with 0.15 g of Se-Met, into each 1 L culture. After incubating the culture with the amino acids for 15 mins, protein expression was induced with 0.1 mM IPTG. The cultures were grown
overnight at 18 °C and the cells harvested by centrifugation and stored at -70 °C.

**Protein purification**

Cell paste was resuspended in 150 mL Buffer A (25 mM HEPES, pH 7.5, 300 mM NaCl, 25 mM imidazole, and 10% glycerol), supplemented with 1 mg/mL lysozyme, 1 mg/mL DNase, 1 mg/mL RNase, 1 mM phenyl methyl sulfonyl fluoride, and 150 μM FAD, and incubated for 30 min with constant stirring. The cells were lysed by sonication at 70% amplitude with cycles of 5s on - 10s off for 5 min. Insoluble material was removed by centrifugation at 30,000 $g$ for 1 hr at 4 °C. The supernatant was loaded onto three in-tandem 5 mL nickel ion-charged IMAC columns equilibrated in Buffer A using an AKTA prime system (GE Healthcare, Chicago, IL). After protein loading was complete, the columns were washed with ~40 mL Buffer A and ~ 20 mL of Buffer A containing 30 mM imidazole. AsFMO was eluted with Buffer B (25 mM HEPES pH 7.5, 300 mM NaCl, 300 mM imidazole, and 10% glycerol). Fractions that contained the MBP-AsFMO fusion protein, as observed by their yellow color, were pooled before adding 6x His-MBP and 6x His-TEV protease by loading the protein mixture onto two 5 mL IMAC columns equilibrated with buffer C (25 mM HEPES pH 7.5 and 100 mM NaCl). The cleaved AsFMO product was separated from the 8x His-MBP protein and 6x His-TEV protease by loading the protein mixture onto two 5 mL IMAC columns equilibrated with buffer C (25 mM HEPES pH 7.5 and 100 mM NaCl). AsFMO was collected in the flow-through, and MBP and TEV protease were eluted with 100% Buffer D (25 mM HEPES pH 7.5, 100 mM NaCl, and 300 mM imidazole). Fractions from the flow-through were yellow, and SDS-PAGE analysis showed a single band corresponding to AsFMO (Fig. S1). These fractions were pooled and concentrated using a 30 kDa centrifuge filter (Sigma Aldrich) and stored at -70 °C in Buffer C. The same method was used to purify SeMet-AsFMO, with the addition of 1 mM TCEP to all purification and storage buffers to prevent SeMet oxidation.

**NADPH oxidation assay**

NADPH oxidation assays were performed in triplicate in 50 mM HEPES, pH 7.5 at 20 °C using an Agilent 8453 spectrophotometer. Assays consisting of varying concentrations of Cys (0-150 mM) and 0.1 mM NADPH were prepared at a volume of 200 μL. To initiate the reaction, 1 μM AsFMO was added immediately before measurements were taken. The change in absorbance at 340 nm was followed for 2 mins.

**Oxygen consumption assay**

The activity of AsFMO was measured using an oxygen electrode system (Hansatech, Amesbury, MA). All assays were performed in 1 mL 50 mM HEPES, pH 7.5, and initiated by the addition of 1-2 μM of enzyme. The steady-state kinetics of AsFMO was determined by varying the concentration of substrate or NAD(P)H (0.01-1.0 mM) with saturated concentrations of Cys (150 mM) at the oxygen air saturation level. The data was fitted to the Michaelis-Menten (equation 1).

$$\frac{v_i}{[E]} = \frac{k_{cat}[S]}{K_M+[S]} \quad (Eq. 1)$$

$[E]$ represents AsFMO concentration, $[S]$ is the concentration of substrate, $k_{cat}$ is the turnover number of the reaction, and $K_M$ is the Michaelis constant.

For data that exhibited substrate inhibition, like NADPH saturation (Fig. 1B), the data was analyzed equation 2. Here, $K_i$ is the substrate inhibition constant.

$$\frac{v_i}{[E]} = \frac{k_{cat}[S]}{K_M+[S]+\frac{[S]}{K_i}} \quad (Eq. 2)$$

The rate constant of hydrogen peroxide production by AsFMO was measured by determining the effect of catalase on the rate of oxygen consumption. It was determined that 1 mg/mL of catalase (Sigma Aldrich) did not decrease or inhibit the activity of AsFMO, by verifying that NADPH oxidation did not change in the presence of catalase (Fig. S5). In an assay containing 1 mg/mL catalase, the rate of oxygen consumption of AsFMO was measured in the
presence of 150 mM Cys, and 25 mM AM, or 150 mM NAC and 0.25 mM NADPH or 0.5 mM NADH, and compared to assays without catalase. The difference between the initial rates was used to calculate the rate of hydrogen peroxide production, taking into account the stoichiometry of the catalase reaction (2 mol H$_2$O$_2$ consumed to 1 mol O$_2$ produced).

**Substrate consumption assay**

To measure the consumption of the amino acid substrates, Cys and SAC, fluorenylmethyloxycarbonyl chloride (FMOC-Cl) and 1-adamantylamine (ADAM) derivatization was used (61). Assays were performed at 200 μL with 15 mM substrate, 5 mM NADPH, and 5 μM AsFMO in 50 mM HEPES, pH 7.5. At different time points between 0.5-5 hrs, 30 μL of the reaction mixture was quenched with 150 μL acetonitrile, and the precipitant removed by centrifuging at 14,000 rpm for 2 minutes. An aliquot of 130 μL of the quenched reaction mixture was then transferred to a conical 96 well plate and 25 μL of 0.2 M borate, pH 8.5 was added. To this solution, 3.25 μL of 50 mM L-ornithine was added as a standard. To initiate derivatization, 10 μL of 158 mM FMOC-Cl prepared in acetonitrile was added. The plate was shaken at room temperature for 5 mins before quenching the reaction with 150 μL of 40 mM ADAM prepared in 50% acetonitrile. The assay was shaken (100 rpm) for 15 minutes and centrifuged before 2 μL of the sample was injected for separation, using an ACCQ-TAG ULTRA C18 column attached to a Waters (Milford, MA) Acquity UPLC equipped with a UV-Vis detector. Samples were monitored at 263 nm. Buffers A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively. Samples were eluted over a gradient of 20-85% Buffer B for 13 mins. The $\text{area}_{\text{substrate}}/\text{area}_{\text{L-ornithine}}$ was calculated using the integrated area under the curve for each sample. Using this analysis, a standard curve from 1-20 mM substrate was constructed and used to measure the change in concentration.

**Product determination with Cys as substrate**

The AsFMO storage buffer (25 mM HEPES, 100 mM NaCl, pH 7.5) was changed using a 10 mL gel filtration column equilibrated in 10 mM ammonium bicarbonate, pH 7.5. All other reagents were prepared in 10 mM ammonium bicarbonate and the pH was adjusted to 7.5, as required. The reaction mixture consisted of 20 mM Cys and 2.5 mM NADPH. The reaction was initiated by addition of 5 μM AsFMO, and then incubated for 2 hrs before removing the enzyme, bypassing the solution through a 10 kDa centrifugal filter. The reaction mix was acidified by adding 1 part 6 N HCl to 100 parts assay to prevent any unwanted cysteine oxidation. Samples were stored at -70 °C until submission to the Virginia Tech mass spectrometry incubator (Blacksburg, VA). All ESI mass spectra were collected by direct injection for accurate mass analysis on an Agilent 6220 TOF LC-MS.

**Detection of sulfenic acid**

Assays were completed with 50 mM 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (62) or cis-5-norbornene-endo-2,3-dicarboxylic acid (norbornene) (63) with 25 μM AsFMO, 2 mM NADPH, and 5 mM Cys. The reaction was incubated for 2 hrs, at which point AsFMO was removed by a 10 kDa centrifugal filter. This assay was performed in 50 mM HEPES at pH 7.5 and ammonium acetate at pH 5.5.

The reaction mix was injected at 10 μL on to a Shimadzu HPLC (Columbia, MD) equipped with a Phenomenex Luna 5 μ C-18 column (Golden, CO). Samples were eluted at a 0.1% water gradient with TFA to 30% acetonitrile with 0.1 % TFA over 20 min, followed by a gradient to 100% acetonitrile, 0.1 % TFA over 20 min, followed by a linear gradient of 100% acetonitrile, 0.1% TFA over 5 min. Dimedone, and any derivatives, were detected at 260 nm and norbornene and norbornene derivatives were detected at 260 nm.

**Size exclusion chromatography**

An AKTA prime plus FPLC equipped with High Prep 16/60 Sephacryl S-200 HR (GE Healthcare) was used to determine the oligomeric state of AsFMO. The column was equilibrated with 50 mM potassium phosphate, pH 7.5, and 100 mM...
NaCl at a flow rate of 1 mL/min. A standard curve was constructed using a high molecular weight marker kit (GE Healthcare, Chicago, IL) supplemented with RNase (Sigma Aldrich, St. Louis, MO) and TEV protease. The curve consisted of ferritin (440 kDa), aldolase (160 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), 6xhis-TEV protease (27 kDa), and RNase (13 kDa). A 500 μL (3 mg/mL) sample of AsFMO was filtered with a 0.22 μm cellulose acetate filter and injected onto the column. For the substrate sample, AsFMO was incubated with 150 mM Cys for 15 minutes prior to injection. AsFMO-containing fractions were verified by 12% acrylamide SDS-PAGE.

**Stopped flow spectrophotometry**

All stopped flow assays were performed with a SX20 stopped flow spectrophotometer equipped with a photodiode array detector from Applied Photophysics (Surrey, UK) housed inside an anaerobic chamber (COY Laboratories, Grass Lake, MI). A detailed protocol of the experimentation has been described previously (64). In brief, the sample handling unit was scrubbed of potential oxygen contaminant overnight using anaerobic buffer solution containing glucose oxidase and dextrose. Protein sample and 1 M Cys stock were deoxygenated with 20 cycles of high vacuum (20 s) and ultra-pure argon (10s). The reducing cofactors were dissolved in anaerobic buffer. All assays were performed in 50 mM HEPES pH 7.5.

For the reductive half reaction, 20 µM of AsFMO was mixed with 20-800 µM NADPH, and spectral changes from 190-850 nm were recorded in a logarithmic scale for 240s (no Cys) or 360s (Cys). AsFMO reduction with Cys was performed by incubating the enzyme with 200 mM Cys prior to sample mixing. AsFMO reduction with NADH was performed by mixing 20 µM AsFMO with 100-1400 µM NADH with spectral changes measured for 180s. The changes at 450 nm during reduction was a best fit with a single (Eq. 3) or double (Eq. 4) exponential decay equation:

\[ \text{Abs} = A_1 e^{-k_{obs1}t} + A_2 e^{-k_{obs2}t} + C \]  
(Eq. 4)

For single exponential decay, \( A \) is the amplitude, \( k_{obs} \) is the observed rate, and \( C \) is the final absorbance. For double exponential decay, the same terms apply with the modification that \( A_1 \) is the amplitude of change that occurs during the first phase, \( A_2 \) is the amplitude change that occurs during the second phase, \( k_{obs1} \) is the observed rate of the first phase, and \( k_{obs2} \) is the observed rate of the second phase. AsFMO reduction with NADPH at 450 nm was a best fit with Eq. 4, while reduction with NADH or in the presence of Cys were a best fit with Eq. 3. Changes at 570 nm during reduction was analyzed using a single exponential rise equation (Eq. 5).

\[ \text{Abs} = A_1 (1 - e^{-k_{obs1}t}) + D \]  
(Eq. 5)

\[ \text{Abs} = A_1 (1 - e^{-k_{obs1}t}) + A_2 (1 - e^{-k_{obs2}t}) + D \]  
(Eq. 6)

The changes at 370 nm best fit with Eq. 5, while changes at 450 nm to Eq. 6. The absorbance at 570 nm exhibited single exponential decay and was analyzed with Eq. 3.

For the oxidative half reaction, a reduced enzyme stock of 20 µM AsFMO mixed with 20 µM NADPH was prepared. An oxygen saturated buffer was prepared following a procedure described previously (64). The reduced enzyme solution was mixed with 400-1100 µM oxygen with measurement being recorded for 300s (no Cys) and 30s (Cys). Changes 370 nm and 450 nm were analyzed using a single (Eq. 5) or double (Eq. 6) exponential rise equation. All similar variables represent the same as described for Eq. 3 and Eq. 4. Variable D represents the initial absorbance of the wavelength.

The concentration dependence of the \( k_{obs} \) values for flavin reduction were analyzed with Eq. 7, where \( k_{obs} \) is the observed rate, \( k_{red} \) is the rate constant for flavin reduction at saturating substrate concentration, \( K_D \) is the dissociation constant for substrate binding.

\[ k_{obs} = \frac{k_{red}[S]}{K_D + [S]} \]  
(Eq. 7)
In order to determine the bimolecular rate constant of C4a-hydroperoxyflavin formation \((k_{\text{OOH}})\), the data were fit to a linear equation. For the experiments that showed minimal rate changes at varying concentration of molecular oxygen \((k_{\text{H2O2}})\) and \((k_{\text{ox}})\), the average of these values was reported.

The kinetic isotope effect (KIE) on the reductive half reaction was determined using isotopically labeled pro\((R/S)-4-{\text{2}}\text{-H-NADPD}. The concentration was kept at 125 μM for the non-deuterated and deuterated cofactors. The kinetic isotope effect was calculated from the ratio of the \(k_{\text{obs}}\) value at 450 for the unlabeled over the labeled cofactor.

**Crystallization**

Crystal conditions for AsFMO were found by screening the conditions in crystal kits 1 and 2 from Hampton Research (Aliso Viejo, CA). Crystals were observed within 4 weeks in 1.6 M ammonium sulfate, 0.1 M NaCl, and 0.1 M HEPES pH 7.5 when AsFMO (4.5 mg/mL) was incubated with 5 mM Cys. Large rod-shaped crystals with a trigonal point were obtained in 1.9 M ammonium sulfate, 0.1 M NaCl, and 0.1 M HEPES with micro-seeding. Droplets were prepared in a ratio of 1: 1.5: 0.5 (AsFMO: mother liquor: micro-seed). All trays were stored at 15 °C after preparation. The same conditions were used to obtain selenomethionine crystals with 1 mM TCEP being present in any enzyme buffer used to prepare the sample. Crystals were grown for ~ 30 days. Crystals were incubated in a solution containing 25% glycerol, 1.8 M ammonium sulfate, 0.1 M HEPES pH 7.5, and 2.5 mM NaCl for 2 min before they were flash frozen in liquid nitrogen.

**X-ray diffraction data collection, phasing, and refinement**

X-ray diffraction data were collected in shutterless mode at NECAT beamline 24-ID-C at the Advanced Photon Source. The data set used for Se-Met SAD phasing consisted of 1800 images covering 360° of rotation. The data set used for refinement consisted of 1000 images covering 200° of rotation. All data sets were integrated and scaled using XDS (65). Intensities were converted to amplitudes using Aimless (66). The space group is \(P3_121\) with the unit cell dimensions of \(a = b = 139.7\ \text{Å}, \ c = 77.9\ \text{Å}\). The asymmetric unit contains one chain of AsFMO. The estimated solvent content is 71%, with a \(V_M\) of 4.25 Å³/Da (67). Data processing statistics are listed in Table 4.

The initial phases were determined by Se-Met single-wavelength anomalous diffraction phasing using SHELXD (68) to identify Se sites, and the structure-determination tools of PHENIX to perform density modification and automated building (69). The calculations identified a constellation of 12 Se sites out of the 14 expected for one protein chain in the asymmetric unit. Density modification with automated model building in PHENIX produced a model consisting of 343 residues with \(R_{\text{work}} = 0.42\) and \(R_{\text{free}} = 0.45\). The model exhibited both α-helices and β-sheets, and these secondary structure elements were arranged in two 3-layer βαα domains as expected for an FMO.

The model from auto-building was the starting point for several rounds of manual building in Coot (70) and refinement against a 2.08Å data set in PHENIX (71). Structure validation was performed using MolProbity and the wwPDB validation service (72,73). Polder maps were used to validate the conformation of the FAD (74). Refinement statistics can be found in Table 4.
Data availability

The coordinates and structure factors have been deposited in the PDB under accession code 6WPU. The authors declare that all other data supporting the findings of this study are available within the article and its supporting information.

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Conflict of interest

The authors state no conflict of interest in relation to this manuscript.

Footnotes

FMO Flavin containing monoxygenase
SAC S-allyl-L-cysteine
Cys L-cysteine
NAC N-acetyl-L-cysteine
AM Allyl mercaptan

Author contributions.
H.V. designed and performed all kinetic experiments and crystal optimization. A.C. obtained and performed the structural analyses. N.S. performed the initial screening of crystallization conditions. H.G.N. established the expression and purification procedures. S.L. performed the gene subcloning. J.P.S. collected structural data. P.S. help with the design and analysis of all biochemical experiments and supervised the project. J.T. analyzed the structural data and supervised the project. H.V. and P.S. wrote the manuscript with contributions from J.T. and A.C.

References


Scheme 1: Generally accepted allicin biosynthetic pathway (5). Feeding studies performed by Granroth have shown serine and glutathione to be key metabolites of this process as they are integrated into SAC (12). AsFMO has been proposed to oxidize SAC, producing the alliin which is subsequently hydrolyzed by alliinase into allyl sulfenic acid and pyruvate. Allyl sulfenic acid then reacts with another allyl sulfenic acid producing allicin.
Scheme 2: Proposed function of AsFMO in allicin biosynthesis.
Scheme 3: Proposed mechanism of AsFMO. Each reaction cycle begins with an oxidized enzyme (A). NADPH tightly binds to AsFMO (B) and transfers the pro-R-C4 hydride of the nicotinamide to the N5 of FAD, producing a flavin hydroquinone (C). The reduced flavin then transfers an electron to oxygen, temporarily producing a flavin semiquinone and superoxide before forming a C4a-hydroperoxyflavin intermediate stabilized by NADP⁺ (D). The substrate (R-S) enters the active site (E) and reacts with the distal -OH group of the C4a-hydroperoxyflavin intermediate through nucleophilic attack producing the hydroxylated product (R-SOH) and a C4a-hydroxyflavin (F). The final step is dehydration of the C4a-hydroxyflavin to regenerate the oxidized flavin and release of products.
Table 1: Steady-state kinetic parameters for AsFMO with different substrates determined by oxygen consumption assay.

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Fixed substrate</th>
<th>$k_{cat}$, s$^{-1}$</th>
<th>$K_M$, mM</th>
<th>$k_{cat}/K_M$, mM$^{-1}$s$^{-1}$</th>
<th>$K_I$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allyl mercaptan (AM)</td>
<td>NADPH</td>
<td>0.75 ± 0.01</td>
<td>1 ± 0.1</td>
<td>0.75 ± 0.08</td>
<td>N.A.</td>
</tr>
<tr>
<td>L-cysteine (Cys)</td>
<td>NADPH</td>
<td>1.3 ± 0.05</td>
<td>11 ± 0.5</td>
<td>0.11 ± 0.007</td>
<td>N.A.</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>NADH</td>
<td>1.7 ± 0.03</td>
<td>5 ± 0.4</td>
<td>0.34 ± 0.03</td>
<td>N.A.</td>
</tr>
<tr>
<td>N-acetyl-L-cysteine (Nac)</td>
<td>NADPH</td>
<td>0.57 ± 0.004</td>
<td>7 ± 0.4</td>
<td>0.08 ± 0.005</td>
<td>N.A.</td>
</tr>
<tr>
<td>NADPH</td>
<td>Cys</td>
<td>1.5 ± 0.02</td>
<td>0.019 ± 0.0002</td>
<td>79 ± 1</td>
<td>4 ± 0.2</td>
</tr>
<tr>
<td>NADH</td>
<td>Cys</td>
<td>1.5 ± 0.01</td>
<td>0.056 ± 0.0002</td>
<td>27 ± 0.2</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Conditions: 50 mM HEPES pH 7.5 with fixed concentrations of 0.25 mM NADPH, 0.5 mM NADH, or 150 mM Cys.
Table 2: Reductive half reaction kinetic parameters of AsFMO in the absence or in the presence of 100 mM Cys.

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>Cofactor</th>
<th>Without Cys</th>
<th>With Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{fast}}$ ($s^{-1}$)</td>
<td>NADPH</td>
<td>7.6 ± 0.3</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>$k_{\text{slow}}$ ($s^{-1}$)</td>
<td>NADPH</td>
<td>0.13 ± 0.003</td>
<td>N.A. $^a$</td>
</tr>
<tr>
<td>$k_{S70}$ ($s^{-1}$)</td>
<td>NADPH</td>
<td>0.050 ± 0.003</td>
<td>N.A.</td>
</tr>
<tr>
<td>$K_{D,\text{fast}}$ (μM)</td>
<td>NADPH</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>$K_{D,\text{slow}}$ (μM)</td>
<td>NADPH</td>
<td>15 ± 2</td>
<td>N.A.</td>
</tr>
<tr>
<td>$K_{D70}$ (μM)</td>
<td>NADPH</td>
<td>33 ± 2</td>
<td>N.A.</td>
</tr>
<tr>
<td>$k_{\text{redfast}}$ ($s^{-1}$)</td>
<td>NADH</td>
<td>4.3 ± 0.03</td>
<td>N.A.</td>
</tr>
<tr>
<td>$k_{\text{redfast}}$ ($s^{-1}$)</td>
<td>NADH</td>
<td>0.70 ± 0.10</td>
<td>N.A.</td>
</tr>
<tr>
<td>$K_{D,\text{fast}}$ (μM)</td>
<td>NADH</td>
<td>84 ± 4</td>
<td>N.A.</td>
</tr>
<tr>
<td>$K_{D,\text{slow}}$ (μM)</td>
<td>NADH</td>
<td>77 ± 5</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Conditions: 50 mM HEPES pH 7.5

$^a$ N.A. indicates not applicable or not determined.
**Table 3:** Oxidative half reaction kinetic parameters of AsFMO in the absence or in the presence of 100 mM Cys.

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>Cofactor</th>
<th>Without Cys</th>
<th>With Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{OOH}} ) (M(^{-1})s(^{-1}))</td>
<td>NADPH</td>
<td>(5500 \pm 400)</td>
<td>(3400 \pm 200)</td>
</tr>
<tr>
<td>( k_{\text{H2O2}} ) (s(^{-1}))</td>
<td>NADPH</td>
<td>(0.002 \pm 0.0005)</td>
<td>N.A. (^a)</td>
</tr>
<tr>
<td>( k_{\text{ox, slow}} ) (s(^{-1}))</td>
<td>NADPH</td>
<td>N.A.</td>
<td>(1.20 \pm 0.08)</td>
</tr>
<tr>
<td>( k_{\text{ox, fast}} ) (s(^{-1}))</td>
<td>NADPH</td>
<td>N.A.</td>
<td>(3.1 \pm 0.01)</td>
</tr>
</tbody>
</table>

Conditions: 50 mM HEPES pH 7.5

\(^a\) N.A., not applicable
<table>
<thead>
<tr>
<th></th>
<th>Phasing</th>
<th>Refinement</th>
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<tr>
<td>Space group</td>
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<td>$P3_121$</td>
</tr>
<tr>
<td>Unit cell</td>
<td>$a = b = 135.49, c = 86.44$</td>
<td>$a = b = 139.72, c = 77.87$</td>
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<tr>
<td>Mols. in asu.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97910</td>
<td>0.97910</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>117.3 – 2.73 (2.86 – 2.73)</td>
<td>121.0 – 2.08 (2.14 – 2.08)</td>
</tr>
<tr>
<td>Observations</td>
<td>492880 (62335)</td>
<td>583633 (38452)</td>
</tr>
<tr>
<td>Unique reflections$^a$</td>
<td>24584 (3179)</td>
<td>52404 (3905)</td>
</tr>
<tr>
<td>$R_{merge}(I)^a$</td>
<td>0.159 (2.940)</td>
<td>0.126 (1.673)</td>
</tr>
<tr>
<td>$R_{meas}(I)^a$</td>
<td>0.163 (3.018)</td>
<td>0.132 (1.765)</td>
</tr>
<tr>
<td>$R_{eom}(I)^a$</td>
<td>0.036 (0.670)</td>
<td>0.039 (0.555)</td>
</tr>
<tr>
<td>Mean I/σ$^a$</td>
<td>20.8 (1.6)</td>
<td>15.6 (1.6)</td>
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<tr>
<td>CC$^{1/2}$</td>
<td>0.999 (0.826)</td>
<td>0.999 (0.642)</td>
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<tr>
<td>Completeness (%)$^a$</td>
<td>99.7 (98.0)</td>
<td>99.7 (96.7)</td>
</tr>
<tr>
<td>Multiplicity$^a$</td>
<td>20.0 (19.6)</td>
<td>11.1 (4.8)</td>
</tr>
<tr>
<td>Anomalous completeness$^a$</td>
<td>99.4 (95.9)</td>
<td>99.6 (94.9)</td>
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<tr>
<td>Anomalous multiplicity$^a$</td>
<td>10.5 (10.3)</td>
<td>5.5 (4.8)</td>
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<tr>
<td>$\Delta_{anom}$ corr. between half-sets$^a$</td>
<td>0.304</td>
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<tr>
<td>No. of protein residues</td>
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<tr>
<td>No. of atoms</td>
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<td>FAD</td>
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<td>water</td>
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<td>$R_{cryst}$$^a$</td>
<td>0.1782</td>
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<tr>
<td>$R_{free}$$^{ab}$</td>
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<td>rmsd bonds (Å)</td>
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<td>rmsd angles (°)</td>
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<td>Ramachandran plot$^c$</td>
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<tr>
<td>Favored (%)</td>
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<td></td>
</tr>
<tr>
<td>Outliers (%)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Clashscore (PR)$^c$</td>
<td>1.3 (100)</td>
<td></td>
</tr>
<tr>
<td>MolProbity score (PR)$^c$</td>
<td>1.15 (100)</td>
<td></td>
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<tr>
<td>Average $B$ (Å$^2$)</td>
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<tr>
<td>Protein</td>
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<td></td>
</tr>
<tr>
<td>FAD</td>
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<td></td>
</tr>
<tr>
<td>water</td>
<td>47.3</td>
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</tr>
<tr>
<td>Coord. error (Å)$^d$</td>
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<td></td>
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<tr>
<td>PDB code</td>
<td>6WPU</td>
<td></td>
</tr>
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</table>

$^a$Values for the outer resolution shell of data are given in parenthesis. $^b$5% test set. $^c$From MolProbity. The percentile ranks (PR) for Clashscore and MolProbity score are given in parentheses. $^d$Maximum likelihood-based coordinate error estimate from PHENIX.
Figure 1: A) Initial velocity determined by monitoring oxygen consumption by AsFMO in 50 mM HEPES pH 7.5 with increasing concentration of substrate SAC (●), Cys (●), NAC (○), and AM (■) in the presence of 0.25 mM NADPH. B) Initial velocity of AsFMO as a function of NADPH (●) and NADH (○) in the presence of 100 mM Cys.
Figure 2: Determination of coupled and uncoupled reaction of AsFMO. The initial velocities of AsFMO as measured by oxygen consumption (black) and the rate of hydrogen peroxide formation (white). The final concentrations were 150 mM Cys, 25 mM AM, 150 mM NAC, 0.25 mM NADPH (*), and 0.5 mM NADH (#). The percentage of the uncoupled reaction for each condition is Cys*=23±3%; Cys#=71±2%; AM*=1±1%; AM#=84±2%; NAC*=35±4%.
Figure 3: Changes in the flavin spectra during reduction with NADPH in the absence (A) or presence of 100 mM Cys (B). Typical reduction at 450 nm is observed in addition to an increase of a long wavelength band with absorbance maximum at 570 nm. Initial spectrum is shown in yellow and the final in brown. Absorbance changes at 450 nm (yellow) and 570 nm (brown). In the absence of 100 mM Cys (C) the absorbance changes at 450 nm best fit to Eq. 4 and at 570 nm to Eq. 5. In the presence of Cys (D), the absorbance at 450 nm best fit to Eq. 3 and at 570 nm to Eq. 5.
Figure 4: AsFMO oxidation. Spectra changes of reduced AsFMO reacted with 300 µM O₂ monitored over 300s in the absence (A) or presence of Cys (100 mM). In panels A and B, the spectra of the reduced flavin is shown in brown, the C4a-hydroperoxyflavin intermediate in blue, and oxidized flavin in yellow. C) Changes in the flavin absorbance at 370 nm (blue, fit with Eq. 5), 450 nm (yellow, fit to Eq. 5) and 570 nm (brown, fit to Eq. 3). D) same as in C but in the presence of 100 mM Cys.
Figure 5: Structure of AsFMO. (A) Fold of AsFMO. The FAD- and NADPH-binding domains are colored pink and blue, respectively. The FAD is shown in yellow spheres. The inset shows the FAD covered by a polder omit map (4σ). (B) Superposition of AsFMO (gray) with pyrrolizidine alkaloid N-oxygenase from Zonocerus variegatus (purple, PDB ID 5NMW). (C) Comparison of the FAD conformations of AsFMO (gray) and pyrrolizidine alkaloid N-oxygenase from Zonocerus variegatus (purple, PDB ID 5NMW). Black dashes indicate an intra-FAD hydrogen bond in AsFMO. Red dashes denote predicted clashes between the FAD of pyrrolizidine alkaloid N-oxygenase and Pro342 in AsFMO.
Figure 6. Models of SAC and AM in the active site of AsFMO. (A) A view into the proposed substrate tunnel. NADP⁺ was modeled based on the structure of FMO from *S. pombe* (PDB code 2GV8). FAD and NADP⁺ are colored yellow and cyan, respectively. (B) Cutaway view of a model of SAC in the substrate tunnel. (C) Cutaway view of a model of AM in the substrate tunnel.
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Hannah Valentino, Ashley C. Campbell, Jonathan P. Schuermann, Nazneen Sultana, Han G Nam, Sophie LeBlanc, John J. Tanner and Pablo Sobrado

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