Characterization of 2-Oxo-3-pentynoate as an Active-Site-Directed Inactivator of Flavoprotein Oxidases: Identification of Active-Site Peptides in Tryptophan 2-Monooxygenase†

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ABSTRACT: 2-Oxo-3-pentynoate has been characterized as an active-site-directed inhibitor of selected flavoprotein oxidases. Tryptophan 2-monooxygenase is irreversibly inactivated in an active-site-directed fashion. The addition of FAD affords no protection from inactivation, whereas the competitive inhibitor indole-3-acetamide fully protects the enzyme from inactivation. The inactivation follows first-order kinetics for at least five half-lives. The rate of inactivation shows saturation kinetics, consistent with the formation of a reversible complex between the alkylating agent and the enzyme before inactivation occurs. Values of 0.017 ± 0.0005 min^{-1} and 44 ± 7 μM were determined for the limiting rate of inactivation and the apparent dissociation constant for 2-oxo-3-pentynoate, respectively. Tryptic maps of tryptophan 2-monooxygenase treated with 2-oxo-3-pentynoate show that two peptides are alkylated in the absence of indole-3-acetamide but not in its presence. The two peptides were identified by mass spectrometry as residues 333−349 and 503−536. Based upon sequence analysis, cysteine 511 and either cysteine 339 or histidine 338 are the likely sites of modification. In contrast, incubation of D-amino acid oxidase or nitroalkane oxidase with 2-oxo-3-pentynoate results in a loss of 55% or 100%, respectively, of the initial activity. In neither case does a competitive inhibitor affect the rate of inactivation, suggesting that the effect is not due to modification of active-site residues.

The flavoprotein tryptophan 2-monooxygenase (EC 1.13.12.3; TMO[1]) from *Pseudomonas savastanoi* catalyzes the oxidative decarboxylation of tryptophan to indole-3-acetamide, with concomitant production of carbon dioxide and water (*1*). This is the first step in a two-step pathway for the biosynthesis of the plant growth hormone indoleacetic acid by a number of bacterial pathogens (*2*, *3*). The high concentration of indoleacetic acid at the site of infection results in production of tumor-like growths referred to as galls (*2*, *3*). TMO has a molecular mass of 62 500 Da, and contains one FAD per monomer. No sequence similarity to any known primary structure is observed outside the putative FAD-binding site. Mechanistically, TMO is similar to the archetypal flavoprotein oxidase D-amino acid oxidase, in that both oxidize an amino acid substrate to the imino acid, transferring electrons to FAD (*4*, *5*). The initial reactions of both reduced enzymes with oxygen also appear to be similar, but TMO then catalyzes the rapid decarboxylation of the imino acid (*6*). Two other flavoproteins, lysine monooxygenase from *Pseudomonas fluorescens* and phenylalanine oxidase from *Pseudomonas* sp. P-501, are also known to catalyze oxidative decarboxylations of amino acids (*7*, *8*). Although extensive kinetic and mechanistic studies of this group of enzymes have been reported (*4*−*6*, *9*, *10*), little structural information beyond the amino acid sequences is available. Chemical modification studies with *N*-ethylmaleimide, methylmethanethiosulfonate, and diethyl pyrocarbonate have suggested the presence of histidine and cysteine residues at or near the active site of TMO (*4*). An arginyl residue, corresponding to arginine 98 of TMO from *P. savastanoi*, is modified when phenylalanine oxidase is inactivated with phenylglyoxal (*11*).

An effective strategy in the design of active-site inhibitors of flavoprotein oxidases has been incorporation of an acetylene moiety into a substrate or product which can react with an active-site nucleophile (*12*, *13*). For example, use of propargylglycine resulted in the identification of His-307 and Tyr-228 as important residues for FAD binding and catalysis in D-amino acid oxidase (*14*−*16*). The acetylenic ketone 2-oxo-3-pentynoate has recently been described as

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[1] Abbreviations: TMO, tryptophan 2-monooxygenase; DAAO, hog kidney D-amino acid oxidase; NAO, nitroalkane oxidase; 2-OP, 2-oxo-3-pentynoate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, *N*,*N*,*N*′,*N*′-ethylendiaminetetraacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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an active-site-directed inhibitor of 4-oxalocrotonate tautomerase (*17*). Since 2-oxo acids are competitive inhibitors of TMO (*4*), 2-OP was examined as a potential active-site-directed inhibitor of TMO. To examine whether 2-OP is generally useful as a probe of the active sites of flavoprotein oxidases, we have also examined the reaction of 2-OP with two other members of this group, D-amino acid oxidase and nitroalkane oxidase. DAAO is a well-characterized enzyme which is often used as a model for this entire group of enzymes (*18*). NAO is a poorly characterized flavoprotein which catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones with concomitant production of nitrite and hydrogen peroxide (*19*). An amino acid residue with a pK_a value of 6.7 has been proposed to act as a base, abstracting the α-proton from the substrate (*20*). The results of these studies are described here.

MATERIALS AND METHODS

Materials. The synthesis of 2-OP has been described elsewhere; the purity of the 2-OP was greater than 95% as assessed by NMR spectroscopy (*17*). FAD, indole-3-acetamide, and L-tryptophan were obtained from Sigma. Sodium [^{3}H]borohydride was purchased from NEN Life Science Products Inc. TPCK-treated trypsin was acquired from Worthington. All other reagents were of the highest purity commercially available. Recombinant TMO from *P. savastanoi* expressed in *E. coli* was purified as described in Emanuele et al. (*4*). D-Amino acid oxidase was purified from pig kidney as described by Fitzpatrick and Massey (*21*). NAO from *Fusarium oxysporum* (ATCC 695) was prepared according to Gadda and Fitzpatrick (*22*).

Methods. The concentrations of native TMO and DAAO were determined spectrophotometrically using extinction coefficients of 11 400 and 11 300 M^{-1} cm^{-1} at 466 and 455 nm, respectively (*4*, *18*). The concentrations of native NAO and of all enzymes after modification were determined by the Bradford method using bovine serum albumin as the standard (*23*). Enzyme activities were measured in air-saturated 0.2 M Tris buffer, pH 8.0, by monitoring the rate of oxygen consumption with a Yellow Springs Instruments Model 5300 Clark electrode. NAO and DAAO were assayed at 30 °C in the presence of 0.3 and 0.07 mM FAD with 2 mM nitroethane or 50 mM DL-alanine, respectively. TMO activity was monitored at 25 °C using 2.5 mM L-tryptophan as substrate. In all cases, 1 unit of enzymatic activity corresponds to the consumption of 1 μmol of oxygen per minute. UV−Visible absorbance spectra were recorded using a Hewlett-Packard Model HP 8453 spectrophotometer equipped with a thermostated water bath. Fluorescence emission spectra were recorded with an SLM Model 8000 spectrofluorometer equipped with a thermostated water bath. Stock solutions of 2-OP were prepared just before use in 0.1 M dibasic sodium phosphate buffer. The addition of 2-OP adjusts the pH to ∼7.0. To monitor inactivation, the enzymes were incubated with the inhibitor under the conditions described in the text. At different times, aliquots were withdrawn and assayed for enzymatic activity. For experiments in which the effect of indole-3-acetamide or benzoate on the rate of inactivation was measured, the enzymes were incubated for 5 min with the competitive inhibitor before the addition of 2-OP. Both indole-3-acetamide and benzoate stock solutions were prepared in 0.2 M Tris buffer prior to use, and the pH was adjusted to 8.0 with HCl or KOH. Gel filtration to remove residual 2-OP after a reaction was carried out on a Sephadex G-25 column equilibrated with 0.2 M Tris buffer, pH 8.0. The irreversibility of 2-OP inactivation of TMO was tested by incubating the modified enzyme isolated by gel filtration for several hours in 0.2 M Tris buffer, pH 8.0, at 25 °C. At different times, aliquots were withdrawn and assayed for enzymatic activity in the presence of 0.6 mM FAD.

To identify the peptides whose modification by 2-OP resulted in enzyme inactivation, ∼40 μM enzyme was incubated with 1.4 mM 2-OP in the presence and absence of 0.19 mM indole-3-acetamide in a total volume of 0.53 mL. The reaction was stopped after 2 h by removing the excess reagent by gel filtration. The modified enzymes were then reacted at room temperature with 2.4 μmol of sodium [^{3}H]borohydride (7.55 Bq/nmol) freshly prepared in water. After 2 h, a further 2.4 μmol aliquot of sodium [^{3}H]-borohydride was added and allowed to react for 15 h. The reaction was stopped by adding trichloroacetic acid to a final concentration of 10% (v/v) and incubating the samples on ice for 30 min. The samples were then centrifuged at 12000*g* for 15 min. Excess reagents were removed by washing the pellets with 10% trichloroacetic acid followed by a chilled acetone/HCl (39:1) mixture. Pellets were resuspended in 0.1 mL of 8 M urea, 4 mM $CaCl_2$, 0.4 M ammonium bicarbonate buffer, pH 8.0, and allowed to stand for 1 h at 37 °C in the dark. The solution was then diluted with water to a final urea concentration of 2 M before the addition of trypsin to a final concentration of 3.3% (w/w). After a 4 h incubation at 37 °C, a second aliquot of trypsin (1.2% w/w final concentration) was added, and the mixture was allowed to react for 15 h at 37 °C. The reaction was stopped with trifluoroacetic acid (1% final concentration). Purification of the peptides was carried out by HPLC using a Waters instrument equipped with a Lambda-Max Model 481 LC Spectrophotometer detector set at 214 nm and a Vydac 218TP54 (4.6 × 250 mm) reverse-phase column at a flow rate of 1 mL min^{-1}. Eluent A was 0.05% aqueous trifluoroacetic acid, and eluent B was 0.04% trifluoroacetic acid in acetonitrile. The chromatography was carried out with 100% A for 5 min, followed by a linear gradient from 0 to 50% B over 90 min. Peptides were collected manually, and the amount of tritium in each was determined by scintillation counting. Radiolabeled peptides were further purified using 5 mM sodium phosphate buffer, pH 6.0, as eluent A and 100% acetonitrile as eluent B. In this case, the elution was from 0 to 25% B in 20 min, then 25% B for 5 min, followed by 25−60% B in 55 min. Automated Edman degradation of purified peptides was carried out on a Hewlett-Packard G1000A protein sequencer at the Protein Chemistry Laboratory of Texas A&M University. Reduction and carboxymethylation were performed directly in the C18 reverse-phase column of a Hewlett-Packard biphasic sample column prior to sequence analysis. The peptide was diluted 1:1 with water and loaded onto a C-18 HPLC cartridge. The cartridge was washed with 1 mL of 0.25 M Tris buffer, pH 8.7, and incubated at room temperature for 30 min with 1 mL of 5 mM dithiothreitol, 0.25 M Tris buffer, pH 8.7, followed by two incubations for 30 min with 1 mL of 100 mM iodoacetamide, 0.25 M Tris buffer, pH 8.7. A 7 mL wash with water and a 2 mL wash with 2% trifluoroacetic acid

were performed before starting the automated sequence analysis.

The molecular masses of the modified peptides were determined by MALDI-TOF mass spectrometry using a Voyager Elite XL mass spectrometer (PerSpective Biosystems, Framingham, MA) at the Laboratory for Biological Mass Spectrometry in the Department of Chemistry at Texas A&M University. Salts were removed from the samples by using a C18 reverse-phase Sep-Pak Plus cartridge (Waters). The peptide was diluted 1:1 with aqueous 1% formic acid and loaded onto the cartridge. After washing the cartridge with 6 mL of water, the peptide was eluted with 0.1 mL aliquots of acetonitrile from 40 to 100% followed by 0.1 mL of 100% acetonitrile. Both radiolabeled peptides eluted at 100% acetonitrile. Samples were prepared for MALDI-TOF mass spectrometry using the overlayer method (*24*). MALDI-TOF mass spectra were acquired in the linear positive ion mode, using α-cyano-4-hydroxycinnamic acid as the matrix.

Data Analysis. The time course of inactivation of TMO by 2-OP was analyzed by fitting the residual activity (A) at a given time (t) to eq 1, where A_0 is the initial activity and k_{obs} is the observed rate of inactivation. The rates of inactivation of the enzyme were fit to eq 2. Here, k_{inact} is the first-order rate constant for the conversion of the reversibly formed enzyme−inhibitor complex to irreversibly inactivated enzyme, K_I is the dissociation constant for the complex, and [I] is the concentration of 2-OP.

$$A = A_0 e^{-k_{obs}t} \quad (1)$$

$$k_{obs} = k_{inact}[I]/([I] + K_I) \quad (2)$$

RESULTS

Inactivation of D*-Amino Acid Oxidase and Nitroalkane Oxidase by 2-Oxo-3-pentynoate.* Treatment of DAAO (41 μM) with 20.3 mM 2-OP in 0.4 mM FAD, 20 mM sodium pyrophosphate buffer, pH 8.5 at 30 °C, produced a slow loss of activity, with a rate of inactivation of 0.0004 min^{-1} (results not shown). The inactivation was not complete, in that no further loss of activity was observed once the activity reached 45% of its initial value. The addition of benzoate (10 mM), a competitive inhibitor of the enzyme with a K_d value of ~3 μM (*25*), did not affect the rate of inactivation. This observation suggested that the inactivation of DAAO by 2-OP is not active-site-directed.

NAO (8 μM) was completely inactivated upon treatment with 16.6 mM 2-OP in 25 mM potassium phosphate buffer, pH 7.0, at 30 °C, with a rate of inactivation of 0.024 min^{-1} (results not shown). A saturating concentration of FAD (0.3 mM)[2] was present during the incubation to prevent formation of apoprotein. The addition of benzoate (30 mM), a competitive inhibitor of the enzyme with a K_i value of 5 mM (*26*), did not affect the rate of inactivation. Thus, the inactivation of NAO by 2-OP is most likely not active-site-directed. No further studies were performed with either NAO or DAAO.

Inactivation of Tryptophan 2-Monooxygenase by 2-OP. Treatment of TMO with 2-OP at pH 7.9 and 25 °C resulted in a time-dependent loss of enzymatic activity (Figure 1A).

[2] The K_M value for FAD is 14 μM at pH 8 (26).

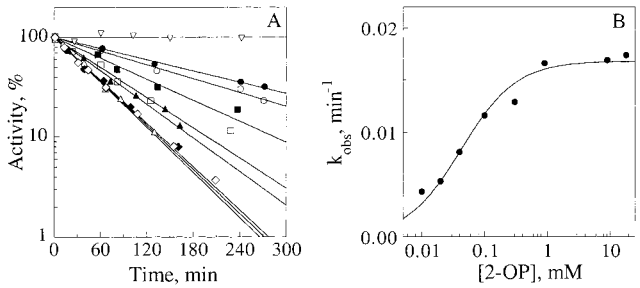


FIGURE 1: Time-dependent inactivation of TMO as a function of 2-OP concentration. TMO was incubated with different concentrations of 2-OP in 0.1 M Tris buffer, pH 7.9, at 25 °C. At different times, aliquots were withdrawn and assayed for enzymatic activity with 2.5 mM L-tryptophan as substrate as described under Materials and Methods. Panel A: Time course of inactivation; concentrations of 2-OP were (●) 0.01 mM, (○) 0.02 mM, (■) 0.04 mM, (□) 0.1 mM, (▲) 0.3 mM, (△) 0.9 mM, (◆) 9.0 mM, (◇) 18.0 mM, and (▽) 17 mM 2-OP in the presence of 0.2 mM indole-3-acetamide. Panel B: Secondary plot of the observed rate of inactivation as a function of the concentration of 2-OP. The line is a fit of the data to eq 2.

Scheme 1

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E_{inact}$$

Indole-3-acetamide, a competitive inhibitor of TMO with a K_i value of 16 μM (*4*), fully protected the enzyme from inactivation (Figure 1A). This result suggests that 2-OP acts at the active site of TMO. The inactivation was monophasic and followed first-order kinetics for at least five half-lives. As shown in Figure 1, the observed rate of inactivation of TMO was dependent on the concentration of 2-OP. A plot of the observed rate of inactivation versus the inhibitor concentration showed saturation kinetics (Figure 1B), suggesting the formation of a reversible complex between 2-OP and the enzyme before inactivation occurs (Scheme 1). The data were well fit by such a mechanism, with a value for the limiting rate of inactivation (k_{inact}) of 0.017 ± 0.0005 min^{-1} and an apparent dissociation constant (K_I) for the reversibly formed complex of 44 ± 7 μM. The ability of 2-OP to function as a substrate for TMO was also tested. No oxygen consumption was observed when 5.6 mM 2-OP was added to an assay mixture containing the enzyme at pH 8.0 and 25 °C.

Properties of TMO Inactivated by 2-OP. Inactivated TMO was isolated by gel filtration after 5 h incubation with 18 mM 2-OP. The resulting enzyme was about 2% active and showed a new absorbance peak at 322 nm (Figure 2). No absorbance maximum was observed above 400 nm, suggesting that no oxidized FAD remained bound to the modified enzyme. No recovery of activity was observed when the inactivated enzyme isolated by gel filtration was incubated for 4 h with 0.6 mM FAD at 25 °C, consistent with the inactivation by 2-OP being irreversible. Enzyme incubated for 5 h with 18 mM 2-OP in the presence of 0.2 mM indole-3-acetamide was also characterized after separation from small molecules by gel filtration. The indole-3-acetamide-protected TMO retained full activity, and FAD was still bound to the enzyme. An absorbance peak at 322 nm was also observed in the spectrum of this sample (Figure 2). However, the relative intensity of the peak at 322 nm was only about 75% that of the enzyme treated in the absence of indole-3-acetamide.

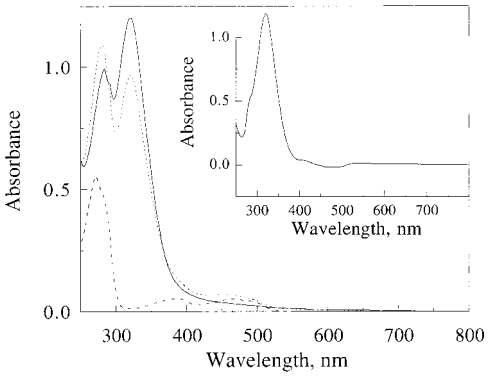


FIGURE 2: Absorbance spectra of TMO after treatment with 2-OP in the presence and absence of indole-3-acetamide. TMO in 20 mM Tris buffer, pH 7.9, was isolated by gel filtration after 5 h incubation with 18 mM 2-OP in the presence and absence of 0.2 mM indole-3-acetamide. The spectrum of native TMO is shown for comparison. All the spectra are normalized to the same protein concentration. Solid line, enzyme treated with 2-OP only; dotted line, enzyme treated with 2-OP in the presence of indole-3-acetamide; dashed line, native enzyme. Inset, difference spectrum of the alkylated TMO minus the untreated enzyme.

The relationship of the loss of FAD to the loss of activity was explored further. The presence of an excess amount of FAD (0.5 mM) during the reaction did not affect the rate of inactivation (data not shown). This suggests that prior formation of the apoenzyme was not a prerequisite for inactivation by 2-OP. The rate of loss of FAD from the enzyme during inactivation by 2-OP was determined directly by measuring the rate of increase of the flavin fluorescence.[3] When TMO (2.2 μM) was incubated in a fluorometer cuvette with 17.3 mM 2-OP in 0.1 M Tris buffer, pH 7.9, at 25 °C, the rate of fluorescence increase was 0.03 min^{-1}, while the rate of inactivation determined in a separate experiment under the same conditions was 0.02 min^{-1}. Thus, FAD loss and inactivation occur at comparable rates. The modified enzyme showed no activity if 0.6 mM FAD was included in assays, so that the modification is not due solely to a decrease in the affinity for the cofactor.

Identification of the Sites of Alkylation of TMO by 2-OP. To identify the peptides whose modification by 2-OP resulted in enzyme inactivation, TMO (20 nmol) was reacted with 1.4 mM 2-OP both in the presence and in the absence of indole-3-acetamide. After 2 h, both reactions were stopped by separating the enzyme and inhibitor on a gel filtration column. The residual activity of the sample incubated in the absence of indole-3-acetamide was ~15% that of the untreated enzyme, while the sample incubated in the presence of indole-3-acetamide was fully active. To identify the sites of modification, both samples were digested with trypsin and the resulting peptide mixtures separated by reverse-phase HPLC. The resulting peptide maps were indistinguishable from one another (results not shown). The 2-OP adduct formed by 4-oxalocrotonate tautomerase was similarly not stable to proteolytic digestion and HPLC separation of peptides (*17*). In that case it was necessary that the adduct be reduced with sodium borohydride to generate a more stable linkage prior to peptide mapping (*17*). A similar approach was taken here with TMO, chemically reducing

[3] The fluorescence emission of FAD bound to TMO is about 1.2% that of free FMN, with maximal emission at 525 nm (4).

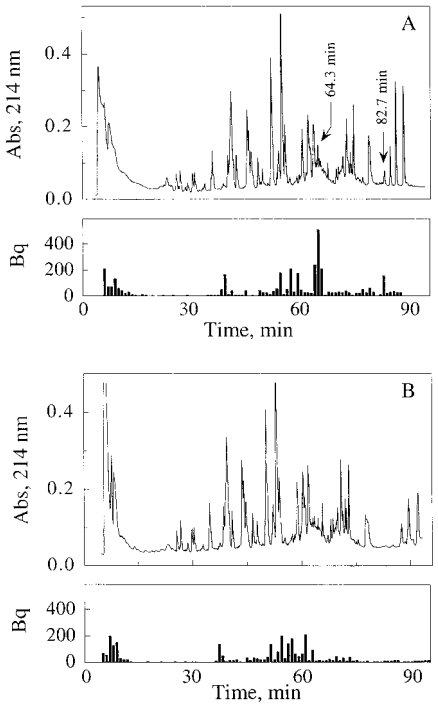


FIGURE 3: HPLC of tryptic digests of TMO modified with 2-OP in the presence and absence of indole-3-acetamide. TMO was incubated with 2-OP in the presence and absence of indole-3-acetamide as described under Materials and Methods. Tryptic digests of each sample were chromatographed as described under Materials and Methods. Peptide elution was monitored at 214 nm. The radioactivity associated with each peptide peak was determined after collection and scintillation counting of individual fractions. The two peptides eluting at 64.3 and 82.7 min found only in the indole-3-acetamide-protected sample are indicated by arrows. (A) No indole-3-acetamide; (B) presence of indole-3-acetamide.

the 2-OP bound to TMO with sodium [^{3}H]borohydride. An additional benefit of such an approach is that the introduction of tritium facilitates the identification of the modified peptides. Accordingly, TMO was modified with 2-OP in the presence and absence of indole-3-acetamide, and both protein samples were treated with sodium [^{3}H]borohydride prior to tryptic digestion and HPLC. Two peptides, eluting at 64.3 and 82.7 min, were present only in the sample prepared in the absence of indole-3-acetamide (Figure 3). Radioactivity coeluted with these two peaks, indicating that they contained a 2-OP adduct. Several other radioactive fractions were found in both samples, consistent with additional modifications of nonessential residues outside the active site.

The two radioactive peptides eluting at 64.3 and 82.7 min in the sample incubated in the absence of indole-3-acetamide were further purified by HPLC at pH 6.0. A single radioactive peak was isolated from each sample (data not shown). These two purified peptides were analyzed by automated Edman degradation and mass spectrometry. The peptide eluting at 82.7 min showed a molecular mass of 3522.4 Da as determined by MALDI-TOF mass spectrometry (data not shown), consistent with a peptide corresponding to the sequence 503−536 of TMO plus a reduced 2-OP moiety. The amino acid sequence of the peptide was determined by automated sequence analysis to be DTGLYLAGXSXSF-AGGWIEGAVQTALNSAXAVLR. Based on the sequence previously determined from the DNA, the X residues correspond to cysteine residues at positions 511, 513, and

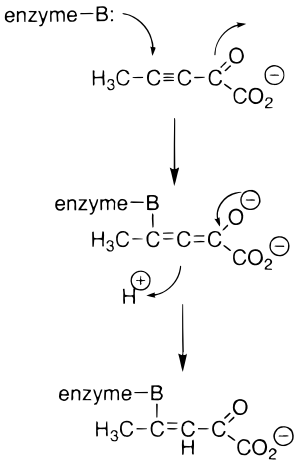
532 (*27*). These are also the most likely sites for alkylation based on the chemical properties of 2-OP (*28*). To distinguish among these three residues, the peptide was treated with iodoacetamide to carboxymethylate unmodified cysteine residues. The Edman analysis was then repeated; in this case the residues at positions 513 and 532 could be identified as carboxymethylcysteine. No carboxymethylcysteine was observed for the residue at position 511. These results are consistent with cysteine 511 being the residue reacting with 2-OP.

The peptide eluting at 64.3 min was resolved into three components upon MALDI-TOF mass spectrometry, with masses of 2084.4, 2100.7, and 2116.6 Da (data not shown). The value of 2084.4 Da is that expected for a peptide corresponding to the sequence 333−349 of TMO containing a reduced 2-OP moiety. This peptide also contains two methionine residues, at positions 334 and 336. The 16-mass unit incremental difference observed between the three peaks in the MALDI-TOF spectrum suggests that the higher mass peaks are due to oxidation of the methionine residues.[4] The sequence analysis confirmed the presence of a single peptide, with the sequence AMQMIHXLTDSESFLSR. This sequence corresponds to the peptide 333−349, with X corresponding to cysteine 339 (*27*), in agreement with the mass spectrometry data. Based on the reactivity of the side chains in this peptide, the most likely site for alkylation within this sequence is either histidine 338 or cysteine 339.

DISCUSSION

In the absence of crystallographic data, one method to identify active-site residues is the use of active-site-directed irreversible inhibitors. Acetylenic and olefinic species have been previously used to identify amino acid residues in the active sites of flavoprotein oxidases (*14*−*16*, *28*). Recently, a new acetylenic ketone, 2-OP, was synthesized and characterized as an inhibitor of 4-oxalocrotonate tautomerase (*17*). That enzyme was completely inactivated by the covalent modification of an active-site residue. The residue was identified as proline 1, the active-site base, initially by mass spectral identification of the modified peptide (*17*) and subsequently by determination of the structure of the modified protein (*29*). The mechanism proposed for the inactivation is shown in Scheme 2. Michael addition of an active-site nucleophile on the acetylenic moiety is followed by subsequent rearrangement to the vinyl keto acid. The reaction is enhanced by polarization of the carbonyl oxygen by an active-site arginine and a solvent molecule. These results suggest that 2-OP would be an effective active-site inhibitor of enzymes which met the following criteria: (1) The active site contains a suitably placed nucleophile. (2) Keto acids bind tightly at the active site, as indicated by their being effective competitive inhibitors. (3) Other residues in the active site are capable of polarization of the carbonyl of the inhibitor. Based upon these criteria, a number of flavoprotein oxidases would appear to be suitable candidates for active-site-directed modification by 2-OP. DAAO has

Scheme 2



seen extensive study over the years as a model system for understanding the mechanisms of simple flavoprotein oxidases. The active site of DAAO contains a conserved arginine residue at position 283 which is proposed to bind the carboxylate of the substrate (*30*, *31*), and the enzyme is inhibited by keto acids (*32*), although the crystal structure does not clearly identify a previously postulated active-site base (*31*). Tryptophan monooxygenase is representative of a related family of flavoprotein oxidases which catalyze both oxidation and decarboxylation of amino acids. Lysine monooxygenase and phenylalanine oxidase are known to catalyze similar oxidative decarboxylations of amino acids to amides (*7*, *8*), but TMO is better understood mechanistically. Keto acids are known to be competitive inhibitors of TMO (*4*), but no three-dimensional structural information is available regarding any of the amino acid oxidase/decarboxylases. However, an arginine in phenylalanine oxidase, which corresponds to arginine 98 of *P. savastanoi* TMO, is modified when the former enzyme is treated with phenylglyoxal (*11*); such a residue could have a similar role to arginine 283 in DAAO. NAO is an apparently unique flavoprotein which oxidizes neutral nitroalkanes. The initial step in catalysis is thought to be formation of a carbanion which presumably is enhanced by polarization of the nitro moiety to allow more ready formation of the nitronate (*26*, *33*). There is no structural information regarding this enzyme. These enzymes were consequently selected as representative in examining the reaction of 2-OP with the broad group of flavoprotein oxidases.

The results presented here show that TMO is indeed irreversibly inactivated by 2-OP in an active-site-directed manner, while the inactivation of NAO and DAAO is not active-site-directed. This conclusion is based upon the effects of competitive inhibitors on the rate of inactivation. Only in the case of TMO does the presence of a competitive inhibitor decrease the rate of inactivation. The saturation kinetic pattern observed for inactivation of TMO is also consistent with an active-site-directed reaction in which 2-OP binds reversibly to the active site prior to the irreversible modification. The K_I value for 2-OP of 44 μM is comparable to the K_m for L-tryptophan of 36 μM (*4*) and presumably contributes significantly to the specificity of the reaction.

The new absorbance peak at 322 nm in the absorbance spectrum of inactivated TMO after gel filtration suggests that the inactivation involves covalent attachment of the inhibitor.

[4] The oxidized methionine residues seen in the MALDI-TOF mass spectrum may have been a consequence of the use of formic acid in sample preparation for mass spectrometry (34). No methionine sulfoxide was seen in the automated sequence analysis of the same peptide which had not been exposed to formic acid.

*

PSESS	506	Leu	**Ile**	**Leu**	**Ala**	**Gly**	**Cys**	**Ser**	**Cys**	**Ser**	**Phe**	Ala	**Gly**	**Gly**	**Trp**	Ile	**Glu**	**Gly**	**Ala**
AGRT3	699	Val	**Ile**	**Leu**	**Ala**	**Gly**	**Cys**	**Ser**	**Cys**	**Ser**	**Phe**	Thr	**Gly**	**Gly**	**Trp**	Val	**Glu**	**Gly**	**Ala**
AGRT4	699	Val	**Ile**	**Leu**	**Ala**	**Gly**	**Cys**	**Ser**	**Cys**	**Ser**	**Phe**	Thr	**Gly**	**Gly**	**Trp**	Val	**Glu**	**Gly**	**Ala**
AGRRA	688	Val	**Ile**	**Leu**	**Ala**	**Gly**	**Cys**	**Ser**	**Cys**	**Ser**	**Phe**	Thr	**Gly**	**Gly**	**Trp**	Val	**Glu**	**Gly**	**Ala**
AGRVI	672	Val	**Ile**	**Leu**	**Ala**	**Gly**	**Cys**	**Ser**	**Cys**	**Ser**	**Phe**	Ser	**Gly**	**Gly**	**Trp**	Val	**Glu**	**Gly**	**Ala**
ERWHE	506	Leu	**Ile**	**Leu**	**Ala**	**Gly**	**Cys**	**Ser**	**Cys**	**Ser**	**Phe**	Thr	**Gly**	**Gly**	**Trp**	Val	**Glu**	**Gly**	**Ala**

FIGURE 4: Comparison of the sequence of the modified peptide eluting at 82.7 min with the sequences of TMO from other sources. *PSESS*, TMO from *Pseudomonas syringae* pv. *savastanoi* (27); *AGRT3*, TMO from *Agrobacterium tumefaciens* plasmid pTiTm-4 (35); *AGRT4*, TMO from *Agrobacterium tumefaciens* plasmid pTiAch-5 (3, 36); *AGRRA*, TMO from *Agrobacterium rhizogenes* (37); *AGRVI*, TMO from *Agrobacterium vitis* plasmid pTiS4 (38); and *ERWHE*, TMO from *Erwinia herbicola* (39). Conserved residues are indicated by boldface type; the asterisk marks the proposed site of alkylation by 2-OP in the *Pseudomonas syringae* enzyme.

* *

PSESS	333	Ala	Met	Gln	Met	Ile	**His**	Cys	**Leu**	Thr	Asp	Ser	Glu	Ser	Leu	Phe	Ser	Arg
AGRT3	529	Asn	Ile	Gln	Leu	Arg	**His**	Cys	**Leu**	Thr	Cys	Asp	Thr	Thr	Ile	Phe	Arg	Ala
AGRT4	529	Asn	Ile	Gln	Leu	Arg	**His**	Cys	**Leu**	Thr	Cys	Asp	Thr	Asn	Ile	Phe	Gln	Ala
AGRRA	515	Asn	Ile	Glu	Met	Arg	**His**	Leu	**Leu**	Thr	Ser	Ser	Asn	Ala	Phe	Phe	His	Ala
AGRVI	501	Asn	Ile	Gln	Leu	Arg	**His**	Leu	**Leu**	Thr	Asn	Asp	Asp	Ser	Phe	Phe	Ser	Tyr
ERWHE	338	Ala	Met	Gln	Met	Val	**His**	Gly	**Leu**	Ser	Ala	Asp	Glu	Thr	Phe	Leu	Asn	Gln

FIGURE 5: Comparison of the sequence of the modified peptide eluting at 64.3 min with the sequences of TMO from other sources. *PSESS*, TMO from *Pseudomonas syringae* pv. *savastanoi* (27); *AGRT3*, TMO from *Agrobacterium tumefaciens* plasmid pTiTm-4 (35); *AGRT4*, TMO from *Agrobacterium tumefaciens* plasmid pTiAch-5 (3, 36); *AGRRA*, TMO from *Agrobacterium rhizogenes* (37); *AGRVI*, TMO from *Agrobacterium vitis* plasmid pTiS4 (38); and *ERWHE*, TMO from *Erwinia herbicola* (39). Conserved residues are indicated by boldface type; the asterisks mark the proposed sites of alkylation by 2-OP in the *Pseudomonas syringae* enzyme.

Such an absorbance peak in the near-UV spectrum is expected for an enzyme species alkylated by an acetylenic compound such as 2-OP (*13*). The mass spectral analyses of the isolated peptides confirm the covalent attachment of the inhibitor. The enzyme is also alkylated by 2-OP to a lesser extent when the competitive inhibitor indole-3-acetamide is present, as shown by the presence of the peak at 322 nm in the absorbance spectrum in this case also. This alkylation appears to involve residues outside the active site, since no loss of activity is seen under these conditions. Further evidence for reactions involving nonessential residues is the labeling of several peptides in both the protected and unprotected samples.

Two peptides were differentially labeled by 2-OP in the presence and absence of indole-3-acetamide. The peptides were identified by amino acid sequencing as residues 333−349 and 503−536. The mass spectral analyses of these peptides are consistent with each containing a single moiety of covalently bound 2-OP. These results place these peptides in the active site of TMO and represent the first identification of potential active site residues in this enzyme. In the case of the 503−536 peptide, direct determination of the amino acid sequence of the modified peptide did not detect any modified residues; however, the three cysteine residues were not seen.[5] These cysteines are the only residues in this peptide which would be sufficiently nucleophilic for reaction with 2-OP (*28*). Both cysteine 513 and cysteine 532 in the modified peptide were still available for reaction with iodoacetamide, ruling out both of these residues as sites of modification. The lack of carboxymethylation of cysteine 511 is consistent with this residue being that modified by 2-OP. The sequence for residues 507−523 is the most conserved region in all the TMO sequences available, with 15 of 17 residues conserved (Figure 4). Cysteines 511 and 513 in *P. savastanoi* TMO are the only two cysteine residues conserved in all the known structures. These two residues are in the middle of a stretch of nine conserved residues. Such conservation is consistent with this region of the protein having a critical functional role such as formation of the active site. Within the second modified peptide, the only two nucleophilic residues are cysteine 339 and histidine 338. Only one of these is modified, based on the stoichiometry determined by mass spectrometry. While the cysteine is not conserved, the adjacent residue, histidine 338, is conserved in all known TMO's (Figure 5). These results therefore are most consistent with either this histidine or this cysteine being the residue whose reaction with 2-OP results in inactivation. Since they are adjacent residues, both are likely to be in or near the active site.

Previous chemical modification studies have shown that TMO can be completely inactivated by cysteine-directed reagents. Both *N*-ethylmaleimide and methylmethanethiosulfonate inactivate TMO (*4*). In the case of *N*-ethylmaleimide, indole-3-acetamide protects the enzyme from inactivation, suggesting that a cysteine residue in the active site is involved. Further evidence for an essential cysteine residue is the need for dithiothreitol in buffers during purification to maintain enzyme stability and prevent loss of the FAD (*4*). The data presented here are consistent with either cysteine 339 or cysteine 511 being the residue responsible

[5] Unmodified cysteines give very low yield in sequencing reactions, so that the absence of a cysteine residue at the expected position in the sequence cannot be taken as evidence that it has been modified.

for these results.

Previous studies have also implicated a histidine residue in TMO as essential. Diethyl pyrocarbonate, which reacts readily with histidine residues, inactivates TMO, and indole-3-acetamide protects the enzyme from inactivation (*4*). In addition, pH−rate profiles of TMO show that a group with a pK_a of 6.0 must be unprotonated for tight binding of amide inhibitors (*6*). The effect of temperature on this pK_a value is consistent with a histidine residue being responsible, as is the pK_a value of 6.0. As noted above, histidine 338 is a conserved residue, and the results presented here make it a candidate for an active-site residue. If histidine 338 is indeed the alkylated residue, it is a reasonable candidate for the histidine identified in the previous studies.

Active-site alkylation of TMO results in the simultaneous loss of the flavin cofactor. The possibility that inactivation requires prior formation of the apoprotein can be ruled out by the lack of any change in the rate of inactivation when 0.5 mM FAD is added to the incubation. The loss of the flavin cofactor must follow alkylation, probably through a structural rearrangement induced by 2-OP incorporation into the active site of the enzyme. During purification of TMO, it is necessary to keep indole-3-acetamide or glycerol present to prevent loss of the cofactor (*4*). This suggests that the FAD is not bound very tightly to the native enzyme. As a consequence, a modification of the active-site residue by a reagent such as 2-OP could readily result in FAD dissociation.

In summary, the results reported in this paper show that 2-OP is an active-site-directed inhibitor of TMO. Two peptides have been shown to be alkylated, placing them in the active site of the enzyme. The modified amino acid residues have been identified as cysteine 511 and either cysteine 338 or histidine 339. This is the first instance in which potential active-site residues have been identified in TMO. These results provide targets for site-directed mutagenesis studies aimed at the elucidation of the roles of these residues in catalysis.

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