Transient Inactivation of Almond Mandelonitrile Lyase by 3-Methyleneoxindole: A Photooxidation Product of the Natural Plant Hormone Indole-3-acetic Acid†

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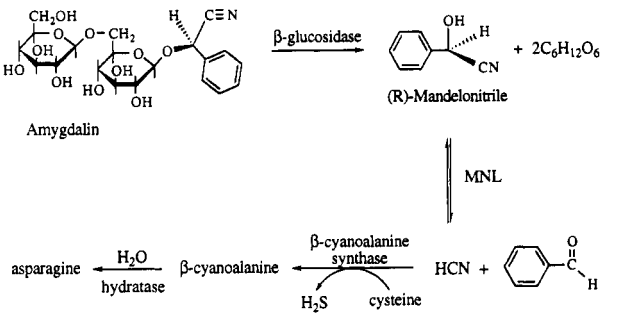
ABSTRACT: A variety of plant growth regulators belonging to the auxin phytohormone family have been found to be good competitive inhibitors of the oxynitrilase from almonds, mandelonitrile lyase (MNL). The major natural auxin, indole-3-acetic acid (IAA), was found to inactivate MNL in a reaction following pseudo-first-order kinetics and dependent upon visible light. Inactivation results from the oxidative decarboxylation of IAA forming 3-methyleneoxindole (MOI). This compound has been synthesized and shown to produce active-site-directed inactivation of MNL, in a reaction following saturation kinetics with a K_I of 37 ± 8 μM and maximal k_{inact} of 0.13 ± 0.02 min^{-1}. Inactivation protection is provided by the competitive inhibitors azide and benzoate, suggesting that the inactivation reaction is active-site-directed. This idea is substantiated by our determination that MOI is a competitive inhibitor of MNL with a K_i of 23 ± 3 μM under steady-state turnover conditions, in reasonable agreement with the value obtained from the inactivation data. Several indole derivatives such as indoline, skatole, oxindole, and 3-methyloxindole are poor competitive inhibitors of MNL with dissociation constants 20–40-fold greater than that for MOI, suggesting a highly specific binding site for the IAA photooxidation product. The enzyme remains inactive following spin dialysis, indicating that a covalent adduct has been formed. However, approximately 30% activity was recovered in a 5-h period following dialysis, and a nearly quantitative recovery occurs in the presence of 2-mercaptoethanol or DTT, indicating that the adduct is labile. The effect of pH on the inactivation reaction suggests modification of a single amino acid functional group with an apparent pK_a of 5.6 ± 0.03. The inactivation data may be explained by a mechanism involving 1,4-conjugate addition of a protein functional group to form an unstable Michael adduct, which is capable of dissociating from the enzyme following dilution or after consumption of excess MOI by added thiols.

Cyanogenic plants are characterized by their ability to produce hydrogen cyanide from the enzymatic degradation of natural products known as the cyanogenic glycosides. These compounds are widely distributed in nature and are found in the tissue of economically important plants such as almonds, sorghum, cassava, and lima beans and in peach, apricot, and wild cherry pits. Despite the ubiquitous distribution of cyanogenic glycosides in nature, their role in plant physiology is not clear (Evered & Harnett, 1988; Ingvorsen et al., 1991; Poulton, 1990; Vennesland et al., 1981).

The metabolism of the cyanogenic glycoside amygdalin through the cyanogenesis pathway is illustrated in Scheme 1. The cyanogenesis pathway is unrelated to the production of cyanide from ethylene biosynthesis in that *S*-adenosylmethionine is the precurosor in the latter pathway (Kende, 1989). The generally accepted function of cyanogenic glycosides is in the protection of plants from predators (Poulton, 1990). However, these compounds may have a more direct role in plant physiology as storage molecules producing carbohydrate and reduced nitrogen for plant growth and development. Through the β-cyanoalanine synthase reaction, the cyanide stored in the cyanogenic glycosides is incorporated into cysteine, forming β-cyanoalanine. This nitrile is subsequently hydrated to asparagine, which itself plays a crucial role in

Scheme 1



plant growth and development as a primary nitrogen transport compound (McGrath & Coruzzi, 1991).

A key step in the cyanogenesis pathway is the oxynitrilase-catalyzed elimination of cyanide from the cyanohydrin precursor. The role of the oxynitrilase reaction is puzzling since cyanohydrins readily dissociate nonenzymatically. However, at the weakly acidic pH of macerated plant tissue (pH 5–6.5), cyanohydrins are relatively stable and it has been suggested that catalysis of cyanohydrin dissociation is required under such conditions (Poulton, 1990). Our work has been focused on the oxynitrilase from bitter almonds, (*R*)-mandelonitrile lyase (*R*-oxynitrilase, EC 4.1.2.10, MNL),[1]

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[1] Abbreviations: MNL, mandelonitrile lyase; FAD, flavin adenine dinucleotide; HLADH, horse liver alcohol dehydrogenase; IAA, indole-3-acetic acid; MOI, 3-methyleneoxindole; RP-HPLC, reverse-phase high-performance liquid chromatography; HMOI, 3-(hydroxymethyl)oxindole; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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which is specific for (*R*)-mandelonitrile as its natural substrate (Scheme 1). MNL is a flavo- and glycoprotein of 75 000 Da with an apparent K_m for D,L-mandelonitrile of 0.59 mM and a maximal turnover number of 37 800 min^{-1} at its pH optimum of 5.5 (Jorns, 1980). A characteristic of all oxynitrilases is the presence of a reactive cysteine residue, which has been identified in mandelonitrile lyase on the basis of its inactivation by thiol-specific reagents (Jaenicke & Preun, 1984; Brush & Yang, unpublished data). The function of this thiol in catalysis has not yet been defined.

The flavoprotein oxynitrilases are quite abundant in plants of the Rosaceae family where they constitute up to 10% of the total soluble protein. The Rosaceae enzymes are also unusual in their requirement for oxidized FAD as a prosthetic group as no apparent redox reaction occurs, and the redox properties of FAD are not involved in catalysis (Vargo et al., 1981). The cofactor is essential as the apoprotein is inactive, but normal activity is recovered upon reconstitution (Jorns, 1979a,b). It has been suggested that the function of the flavin cofactor may be to maintain the active structure of the enzyme or to regulate enzyme activity *via* its redox state (Jorns, 1979b).

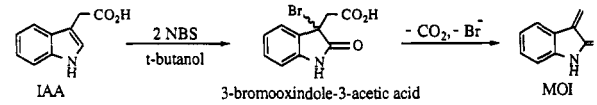
Cyanogenic plants such as cassava, sorghum, and lima beans play an important role as food products for humans and animals in many parts of the world and as a result are responsible for many cases of cyanide poisoning (Poulton, 1983). It would obviously be desirable to develop plant varieties of low cyanogenic potential by mutagenesis, genetic engineering, or selective breeding but without affecting key metabolic pathways or the nutritional value of the plant. In order to accomplish this, a thorough understanding of the enzymology involved in the biosynthesis and metabolism of cyanogenic glycosides is required. Our goal is to examine the mechanistic and structural relationships between the oxynitrilases from different plant species in order to obtain a clear picture of their metabolic function, as well as possible evolutionary relationships.

We have recently characterized an anion recognition site in almond MNL and determined that several auxin plant growth regulators are effective inhibitors of the enzymatic reaction (Brush and Yang, unpublished data). Our interest in the role the oxynitrilase reaction may play in the cyanogenesis pathway has prompted us to investigate in detail the interaction between MNL and these key phytohormones. In this paper we report that almond mandelonitrile lyase undergoes light-dependent inactivation by the natural auxin indole-3-acetic acid. The photooxidation product, methyleneoxindole, has been synthesized and shown to produce active-site-directed transient inactivation of MNL. The inactivation reaction has been initially characterized, and possible mechanisms are discussed.

EXPERIMENTAL PROCEDURES

General. Unless otherwise noted all chemicals, reagents, and buffers were of the highest quality available and were used without further purification. Indole-3-acetic acid and DTNB were from Sigma. Indole, indoline, oxindole, and skatole were purchased from Aldrich. Technical-grade D,L-mandelonitrile was purchased from Aldrich and purified by extracting an ethereal solution with 10% sodium bisulfite to remove contaiminating benzaldehyde. Purified samples were stored under N_2 at −20 °C prior to use. Almond mandelonitrile lyase was purchased from Sigma (product no. M 6782) as an ammonium sulfate suspension. Prior to use, the enzyme (ca. 22 mg in 1 mL) was dialyzed overnight against 0.1 M citrate buffer, pH 5.5, at 4 °C to remove imidazole buffer and ammonium sulfate. Following dialysis, the enzyme preparation had a maximal specific activity of ca. 190 units/mg of protein, consistent with the estimated purity of 90% based on SDS–PAGE (Laemmli, 1970). One unit is equivalent to the amount of enzyme required to produce 1 μmol of benzaldehyde and HCN/min at pH 5.5 and 25 °C. The dialyzed enzyme (ca. 6 mg/mL) was stored at 4 °C, where it lost 20% of its activity over 1 month. Protein concentrations were determined by the Bio-Rad protein dye binding assay (Bradford, 1976), using bovine serum albumin as standard. Enzyme assays were measured in a Perkin-Elmer λ6 UV–vis spectrophotometer with UV Data Manager software and a thermostated cell holder maintained at 25 ± 0.2 °C. Reversed-phase HPLC was carried out using a Rainin solvent delivery system Model HPXL, detector Model 288 set at 254 nm, and Rainin C-18 Dynamax-60A column. A mobile phase of 35% acetonitrile/0.1% trifluoroacetic acid was used throughout, at a flow rate of 1.0 mL/min. NMR spectra were obtained using a Bruker AM-300 spectrometer. Electron impact ionization mass spectra were obtained by Mr. Leo Kenny on a Hewlett-Packard GC–MS.

Scheme 2



Oxindole Synthesis: (*A*) *3-Methyleneoxindole.* Authentic 3-methyleneoxindole was prepared *via* the conversion of indole-3-acetic acid to 3-bromooxindole-3-acetic acid with *N*-bromosuccinimide and subsequent decarboxylation and elimination of bromide (Scheme 2). Our procedure closely followed that reported by Hinman and Bauman (1964a,b), and is reported here in its entirety. To a stirred solution of 6.55 g (0.037 mol) of indole-3-acetic acid (IAA) in 245 mL of anhydrous *tert*-butyl alcohol under nitrogen was added 13.35 g (0.075 mol) of *N*-bromosuccinimide over a 1-h period, while the reaction temperature was maintained between 21 and 23 °C. After 80 min the reaction mixture was concentrated under reduced pressure at room temperature to a thick syrup, which was taken up in 100 mL of anhydrous ether. White crystals of succinimide were removed by gravity filtration and washed with 60 mL of ether. The combined ether filtrates were concentrated to approximately 50 mL at room temperature and reduced pressure to produce a second crop of succinimide, which was removed by filtration. Evaporation of the filtrate produced a syrupy residue; this was repeatedly triturated with benzene and the solvent evaporated *in vacuo* until the mass solidified. The solid residue was suspended in 75 mL of benzene, collected by vacuum filtration, and washed twice with 20 mL of benzene. 3-Bromooxindole-3-acetic acid was isolated as a yellow powder in a yield of 4 g (40.5%). The structure of the product was confirmed by its ^1H NMR spectrum: (DMSO-d_6) δ 3.58 (dd, 2 H, CH_2COOH), 6.92–7.59 (m, 4 H, ArH), 10.79 (s, 1 H, NH). Formation of 3-methyleneoxindole from 3-bromooxindole-3-acetic acid was conducted under dilute conditions in order to avoid the reported polymerization of MOI which occurs in concentrated solution (Hinman & Bauman, 1964b). 3-Bromooxindole-3-acetic acid (0.59 g, 2.2 mmol) was dissolved in 15 mL of 95% ethanol and diluted with 260 mL of deionized water. This solution was immediately extracted with 4 × 50-mL portions of chloroform. The chloroform extracts were combined, washed with 4 × 50 mL of water, and dried over sodium sulfate. Following evaporation of solvent and drying overnight *in vacuo*, 3-methyleneoxindole was obtained as a yellow solid in a yield

of 0.11 g (35%). The identity and purity of the final product was confirmed by its ^{1}H NMR spectrum: (DMSO-d_6) δ 6.24 (d, 2 H, CCH_2), 6.83–7.63 (m, 4 H, ArH), 10.52 (s, 1 H, NH). The absorption spectrum of MOI was determined in 0.1 M citrate buffer, pH 5.5, and is characterized by a double maximum at 248 and 253 nm, with extinction coefficients of 14 820 and 14 250 M^{-1} cm^{-1}, respectively. These values compare well with the extinction coefficients of 23 500 M^{-1} cm^{-1} (ϵ_{248nm} = ϵ_{253nm}) measured by Hinman and Bauman (1964b), which were determined in 95% ethanol. The product was further characterized by its electron impact mass spectrum (methanol). Calculated monoisotropic mass for C_9H_7NO: 145.05 *m/e* found: 145.05 (M^+). Reverse-phase HPLC of authentic MOI indicated the presence of a single component with a retention time of 7.0 min, suggesting greater than 95% purity. 3-Methyleneoxindole was stored desiccated as a dry solid at 0–5 °C, where it is stable for at least 2 months. Aqueous, buffered solutions of MOI were prepared daily, with concentrations determined on the basis of dry weight. The stability of MOI in 0.1 M citrate buffer, pH 5.5, was monitored by UV–visible spectroscopy and RP-HPLC and indicated that the compound remains unchanged over a 9-h period at 25 °C.

The Michael addition compound between *N*-acetyl-L-cysteine and MOI was prepared by mixing equimolar concentrations of each in 0.1 M citrate buffer, pH 5.5. The addition reaction was monitored by following the decrease in the MOI absorbance bands at 248 and 253 nm and was judged to be complete in 2 h. Analysis of the reaction mixture by reverse-phase HPLC indicated the absence of MOI (t_R = 7.0 min) and the appearance of the adduct at 3.2 min.

(*B*) *3-Methyloxindole.* The synthesis of 3-methyloxindole from skatole was similar to that described for 3-bromooxindole-3-acetic acid but required 1 equiv of *N*-bromosuccinimide (Hinman & Bauman, 1964a). The isolated compound has an absorption spectrum in 0.1 M citrate buffer, pH 5.5, characterized by a maximum at 249 nm (ϵ_{249nm} = 7500 M^{-1} cm^{-1}) and a shoulder at 278 nm (ϵ_{278nm} = 1500 M^{-1} cm^{-1}), in good agreement with the literature values obtained in 95% ethanol (Hinman & Bauman, 1964a). The structure of the product was confirmed by its ^{1}H NMR spectrum [(DMSO-d_6) δ 1.35 (d, 3 H, CHCH_3, 3.43 (quartet, 1 H, CHCH$_3$), 6.78–7.28 (m, 4 H, ArH), 10.35 (s, 1 H, NH)] and its electron impact mass spectrum (acetone) [calculated monoisotropic mass for C_9H_9NO: 147.07. *m/e* found: 147.05 (M^+)].

Enzyme Activity Assay. Mandelonitrile lyase activity was measured spectrophotometrically by following the formation of benzaldehyde from D,L-mandelonitrile at 249 nm, using an extinction coefficient of 13 200 M^{-1} cm^{-1} (Jorns, 1980). All activity assays were conducted at 25 °C in 0.1 M citrate buffer, pH 5.5, containing 0.03 unit (0.13 μg) of MNL. After a 1-min preincubation, reactions were initiated by the addition of enzyme. The rate of the apparent enzymatic reaction was corrected for the nonenzymic decomposition of mandelonitrile, which was usually 5–10% of the enzymatic reaction rate. Initial rates were calculated over the initial 10–20% of reaction from the slope of the absorbance vs time curve using the Kinetics utility of the UV Data Manager Software.

Enzyme Inactivation Kinetic Studies. Time-dependent inactivation experiments were conducted by incubating MNL (1.3 units) and an appropriate concentration of 3-methyleneoxindole at 25 °C in 0.1 M citrate buffer, pH 5.5. At various time intervals, 20-μL (0.13-μg) aliquots were removed and diluted into cuvettes containing 0.5 mM D,L-mandelonitrile, and the residual MNL activity was measured. In all cases, the inhibitor was diluted at least 50-fold, where it had no effect on enzyme activity under turnover conditions. MOI stock solutions were freshly prepared in HPLC-grade methanol just before each experiment. A control reaction contained MNL and a suitable aliquot of methanol in place of MOI. The pseudo-first-order rate constants for the inactivation reaction, k_{obs}, were determined by linear regression analysis from plots of log (% remaining activity) vs time.

Light-Induced Inactivation of MNL by IAA. MNL (1.3 units) was incubated with 1 mM IAA in 0.1 M citrate buffer, pH 5.5, (a) in the dark, and (b) while being illuminated by a 600-W slide projector incandescent lamp from a 15-cm distance. The illuminated sample was contained in a 7.5-in. crystallization dish containing deionized water and backed with white filter paper. The temperature was maintained at 25 ± 2 °C during the incubation period. Appropriate controls were prepared containing only enzyme and buffer. Aliquots from reaction mixtures were periodically removed to determine residual MNL activity and to monitor the formation of photodecomposition products by RP-HPLC as described above.

Competitive Inhibition Studies. Reversible inhibition of MNL was determined using the normal activity assay mixture containing 0.2–0.6 mM D,L-mandelonitrile and different, fixed concentrations of inhibitor. Initial velocity kinetic data were analyzed using the nonlinear least-squares program KinetAsyst (Cleland, 1979). In come cases the high UV absorbance of the competitive inhibitors precluded the use of the standard MNL activity assay at 249 nm. An alternative assay was developed based on monitoring the formation of benzaldehyde using HLADH. These assays were conducted in 0.1 M citrate buffer, pH 5.5, although stock solutions of HLADH and NADH were prepared in 0.1 M phosphate buffer, pH 7.2, because of the slow inactivation of HLADH and decomposition of NADH at pH 5.5. The inhibitor being tested was precincubated with 0.03 unit of MNL for 1 min prior to the addition of NADH (0.35 mM), and HLADH (0.3 unit). Reaction was initiated by the addition of D,L-mandelonitrile to the appropriate concentration. The HLADH-catalyzed reduction of benzaldehyde from the MNL-mediated processing of mandelonitrile was determined by the concomitant oxidation of NADH at 340 nm, using an extinction coefficient of 6220 M^{-1} cm^{-1}. Under these conditions, the MNL inhibitors had no effect on the rate of benzaldehyde reduction by HLADH.

Reactivation of MOI-Treated Enzyme. MNL (1.3 units) was incubated with 0.1 mM MOI in 0.1 M citrate buffer, pH 5.5, at 25 °C for 2 h or until <10% of the original activity remained. Under these same conditions, an aliquot of β-mercaptoethanol or DTT was added directly to the inactivation reaction mixture to a final concentration of 1 mM, and the recovery of MNL activity was monitored as a function of time. A control sample was treated similarly, except that MOI was omitted.

Thiol Content of MOI-Treated MNL. The free sulfhydryl content of MNL was determined before and after reaction with MOI using Ellman's reagent, DTNB (Ellman, 1959). In a typical experiment, 6 mg (1200 units) of MNL was incubated with 1.8 mM MOI overnight at room temperature. Excess reagent was removed by washing the protein sample three times in citrate buffer, pH 5.5, using Amicon Centricon 30 microconcentrators. A sample containing 450 μg of protein was added to a cuvette containing 0.1 M phosphate buffer, pH 7.3, 6 M guanidine hydrochloride, 1 mM EDTA, and 0.4 mM DTNB. The absorbance at 412 nm was subsequently monitored over a 40–60-min period. A control sample was carried out in the same manner, except that MOI was excluded,

Table 1: Reversible Inhibition of MNL by Natural and Synthetic Auxin Phytohormones[a]

compound	% inhibition[b]	K_i(μM)
Natural Auxins		
3-methyleneoxindole[c]	46[d]	23 ± 3
indole-3-acetic acid	10	780 ● 110
indole-3-butyric acid	23	291 ± 44
Auxin Biosynthetic Precursors		
D- or L-tryptophan[e]	<5	ND[f]
indole-3-acetonitrile	8	ND
tryptophol	20	ND
Synthetic Auxins		
phenylacetic acid	69	18 ± 2
(2,4-dichlorophenoxy)butyric acid	52	26 ± 5
(2,4-dichlorophenoxy)acetic acid	50	27 ± 6
phenoxyacetic acid	33	126 ± 16

[a] Complete assay conditions are described under Experimental Procedures. [b] Relative inhibition determined using 0.5 mM D,L-mandelonitrile, and 0.1 mM inhibitor unless noted otherwise. [c] Presented for comparative purposes only. [d] Determined using 50 μM MOI. [e] Determined using 4 mM tryptophan. [f] ND, not determined.

and appropriate blanks were employed to account for the background absorbance of MNL and DTNB at 412 nm. The thiol content was calculated from an average of three determinations, using an extinction coefficient of 13 700 M^{-1} cm^{-1} (Riddles et al., 1979) and assuming a monomeric molecular weight of 75 000 (Jorns, 1980).

Effect of pH on MNL Inactivation by MOI. The pH dependence on the inactivation of MNL by MOI was studied at 25 °C utilizing the following buffers: pH 4.5, 50 mM citrate; pH 5.0, 40 mM citrate; pH 5.5, 25 mM citrate; pH 6.0, 20 mM citrate; pH 6.5, 75 mM phosphate; pH 7.5, 50 mM phosphate; and pH 8.0, 40 mM phosphate. Ionic strength was maintained at 0.1 M with NaCl, and the pH values were checked and recorded at the end of each experiment. The concentration dependence was determined at each pH and the kinetic data were analyzed using the nonlinear least-squares program KinetAsyst (Cleland, 1979).

RESULTS AND DISCUSSION

Inhibition of MNL by Auxin Phytohormones. We have identified an anion recognition site on MNL, based on the irreversible inactivation by phenylglyoxal and the competitive inhibition by a variety of aromatic carboxylic acids (Brush and Yang, unpublished data). During the course of those studies, it was noted that several of these inhibitors are natural products in plants, with growth regulatory functions. Given our interest in examining the physiological role of the MNL reaction and the cyanogenesis pathway in plant metabolism, we expanded this study and evaluated a variety of plant growth regulators as potential inhibitors of MNL. These results are summarized in Table 1.

Of the five known classes of growth regulators naturally synthesized by plants, only those containing a carboxylic acid functional group were tested as inhibitors: the auxins, gibberellins, and abscisic acid (Davies, 1987). Of these, only compounds belonging to the auxin family of phytohormones were found to inhibit the MNL reaction. The auxins are known to cause a wide range of physiological effects in plants, such as stem cell enlargement, phototropism, root initiation, flowering, and fruit ripening (Davies, 1987). However, as Table 1 indicates, the natural auxins were found to be only modest competitive inhibitors, and the auxin biosynthetic precursors had little effect on MNL activity at all. The synthetic auxins were an exception as these compounds are particularly effective competitive inhibitors of MNL. These synthetic compounds have auxin-like activity in plants and are typically used in herbicides (Cobb, 1992). The best known are the halogenated derivatives of phenoxyalkanoic acids, which are shown in Table 1 to have micromolar K_is. These results suggest a high affinity for the enzyme active site, possibly due to nonpolar interactions with the aromatic ring and ionic interactions between the carboxylate group and the anion recognition site on the enzyme.

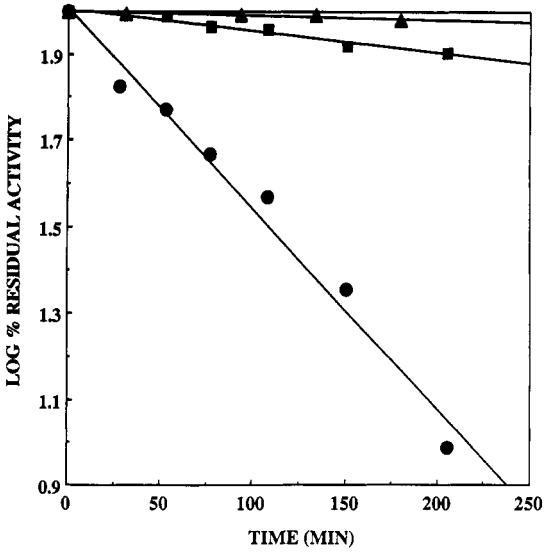


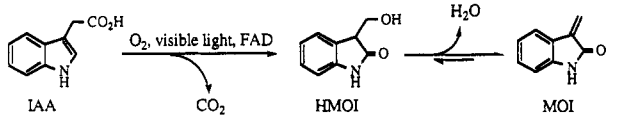
FIGURE 1: Light-induced time-dependent inactivation of MNL by 1 mM IAA. The inactivation reaction mixtures contained 0.1 M citrate buffer, pH 5.5, 1.3 units of MNL, and IAA-visible light (●), IAA-dark (▲), or visible light only (■).

Light-Dependent Inactivation of MNL by IAA. It is tempting to speculate whether or not the auxins, especially the synthetic compounds, may have an effect on the oxynitrilase reaction *in vivo*. It was noted that the natural phytohormone IAA is a relatively poor competitive inhibitor, with a K_i almost 10^3-fold greater than the estimated micromolar concentration of IAA in plant tissues (Cobb, 1992). However, extended incubation of IAA with MNL, or incubation with an IAA solution exposed to fluorescent lights for 1 week, resulted in enzyme inactivation. This was a surprising result as we did not expect IAA to participate in nucleophilic or electrophilic reactions with protein functional groups, although we were aware of the sensitivity of indoles to photodecomposition by visible light. Incubation of MNL with 1 mM IAA at pH 5.5 and irradiation of the sample with visible light resulted in a slow, first-order, time-dependent loss of enzyme activity with k_{obs} = 0.011 min^{-1} (Figure 1). The irradiated sample lost 90% of its initial activity after 200 min, while control samples containing enzyme incubated in the absence of IAA, or with 1 mM IAA in the dark, lost only 15% activity over the same time period.[2]

The illuminated reaction mixture was periodically analyzed by RP-HPLC. In addition to the elution of IAA at 6.0 min, a new compound was detected during the course of the reaction, eluting at 3.0 min. As the reaction continued, this polar compound began to decrease in concentration, concomitant with the formation of a nonpolar product with a retention

[2] At this time it it not clear if the photodecomposition of IAA in the presence of MNL is mediated by protein-bound flavin or by free FAD which has dissociated from the enzyme. Preliminary studies suggest that MNL does accelerate the rate of IAA photooxidation and that the rate enhancement is sensitive to active-site-directed inactivation of MNL by phenylglyoxal. More detailed studies are currently in progress.

Scheme 3



time of 7.0 min. At the end of the experiment, the absorption spectrum of the irradiated reaction mixture (data not shown) suggested that the indole ring of IAA had been modified as indicated by the formation of new UV absorbance bands at 248 and 253 nm. The appearance of these absorbance maxima near 250 nm is a characteristic of oxindoles which contain an exocyclic double bond at the C-3 position (Hinman et al., 1961). These results indicated that the chemical species responsible for the observed inactivation of MNL was not IAA itself but possibly a photodecomposition product.

The photooxidation chemistry of IAA has been studied in some detail (Fukuyama & Moyed, 1964; Still et al., 1965; Amat-Guerri et al., 1990), and it is known that the reaction is mediated by ultraviolet light, or visible light in the presence of a flavin sensitizer. The major, nonpolymeric products were identified as oxindole derivatives resulting from the photo-induced oxidative decarboxylation of IAA to produce 3-hydroxymethyloxindole and 3-methyleneoxindole (Scheme 3). The equilibrium favors dehydration to form 3-methyleneoxindole, which is typically observed as the major product (Still, et al., 1965). A similar reaction also occurs in plants by the action of peroxidase on IAA as part of the oxidative decarboxylation pathway for IAA catabolism (Hinman & Lang, 1965; Nakono et al., 1982; Kobayashi et al., 1984). 3-Methyleneoxindole has been reported to stimulate plant growth as a auxin phytohormone (Tuli & Moyed, 1969); however, this suggestion is controversial (Anderson et al., 1972; Evans & Ray, 1972; Evans, 1976). Since these photodecomposition products inhibit the growth of higher plants, have potent bacteriostatic activity, and inhibit alcohol dehydrogenase, isocitrate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase (Still et al., 1965), we have undertaken the synthesis of MOI (Hinman & Bauman, 1964a,b) in order to evaluate its effect on the MNL-catalyzed reaction.

Inactivation of MNL by 3-Methyleneoxindole. 3-Methyleneoxindole was prepared as a yellow solid of greater than 95% purity, based on its analysis by RP-HPLC and ^{1}H NMR. Authentic MOI coelutes with the nonpolar product from the photodecomposition of IAA (t_R = 7.0 min), suggesting that the more polar compound eluting at 3 min is 3-hydroxymethyloxindole, which dehydrates forming MOI. Incubation of MOI with MNL at pH 5.5 results in pseudo-first-order, time- and concentration-dependent loss of enzyme activity Figure 2.

The addition of benzoate or azide, competitive inhibitors of the MNL reaction (Jorns, 1980), resulted in inactivation protection from MOI. The pseudo-first-order rate constant for inactivation by 0.1 mM MOI was 0.085 min^{-1}, whereas in the presence of 5 mM azide or 0.1 mM benzoate the rate constants decreased to 0.013 and 0.019 min^{-1}, respectively. The fact that a 50-fold higher concentration of azide provides protection against inactivation comparable to that provided by benzoate is consistent with the 100-fold difference in the K_is between these compounds (Jorns, 1980). As the degree of inactivation protection is consistent with the relative affinity of each compound for MNL, the inactivation reaction by MOI is probably occurring at or near the active site. This idea is consistent with the good fit of the primary inactivation rate

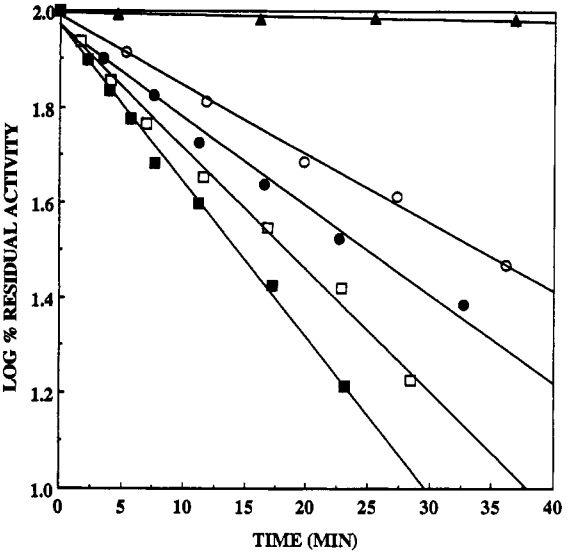


FIGURE 2: Time- and concentration-dependent irreversible inhibition of MNL by MOI. Inactivation reaction mixtures contained 0.1 M citrate buffer, pH 5.5, 1.3 units of MNL, and the following concentrations of MOI: 60 μM (■), 28 μM (□), 18 μM (●), 13 μM (○), and 0 μM (▲).

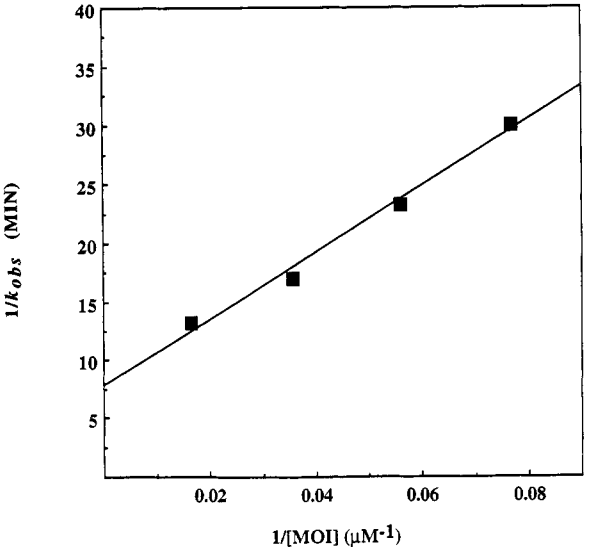


FIGURE 3: Double-reciprocal plot of k_{obs} *vs* MOI concentration (from Figure 2), indicating saturation kinetics in the inactivation of MNL by MOI.

and concentration data to eq 1, as shown in Figure 3.

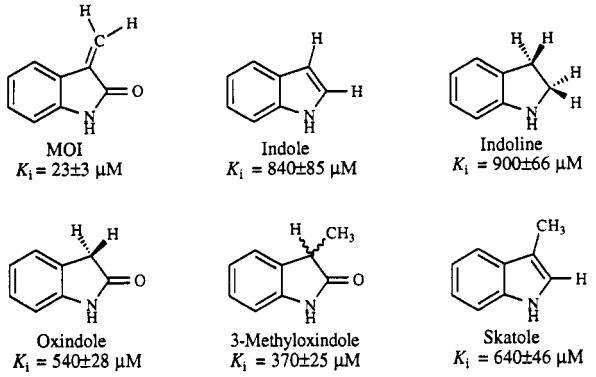
$$1/k_{obs} = 1/k_{inact} + (K_I/k_{inact})\ (1/I) \qquad (1)$$

Equation 1 predicts hyperbolic saturation kinetics (Kitz & Wilson, 1962; Plapp, 1982), indicating that the inactivation of MNL by MOI proceeds through the initial formation of a reversible enzyme-inhibitor complex prior to the covalent modification event. An apparent K_I of 37 ± 8 μM and maximal inactivation rate, k_{inact}, of 0.13 ± 0.02 min^{-1} were calculated from the fit of the data to eq 1. Equation 2 describes the reaction between an enzyme and an active-site-directed affinity reagent (Meloche, 1967), where E·I is the MNL–MOI complex, and E-I′ is covalently modified enzyme.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E\text{-}I' \qquad (2)$$

The K_I in this equation is defined as $(k_{-1} + k_2)/k_1$ and represents the concentration of MOI giving the half-maximal

Chart 1



inactivation rate, k_{inact}, and presumably half-saturating the enzyme (Meloche, 1967). This definition differs from that for the K_i of a reversible inhibitor, which is a measure of the dissociation constant of the enzyme–inhibitor complex (Silverman, 1988). A direct comparison of the K_I value obtained from inactivation kinetic data with the K_i determined by measuring the effect of an inhibitor on the enzyme–substrate complex is possible only if k_2 in eq 2 is much smaller than k_1 or k_{-1}. Verification that the K_I determined from the concentration dependence on MNL inactivation by MOI is an accurate measure of the active-site affinity of the inhibitor was accomplished by determining the K_i for MOI following standard competitive inhibition kinetics under steady-state turnover conditions. The inhibition rate data were fit to eq 3, resulting in the determination of a competitive K_i of 23 ± 3 μM, in reasonable agreement with the K_I from inactivation kinetics.

$$V = V_{max}S/(K_m(1 + I/K_i) + S) \quad (3)$$

Characterization of the MOI Binding Site. The inactivation kinetic data indicate that MNL has a high affinity for MOI, with a K_m/K_i ratio of approximately 25, based on the reported K_m of 590 μM for D,L-mandelonitrile (Jorns, 1980). This result was somewhat surprising given that MOI and mandelonitrile share little structural similarity, other than for the nonpolar aromatic ring. There are no structural data available which describe the interactions of the indole or oxindole group with the active-site region of MNL. This information is of critical importance in explaining the competitive inhibition of MNL by the auxin phytohormones and for the design of mechanistic probes to help elucidate the inactivation by MOI. Several indole derivatives were tested as competitive inhibitors of MNL using the HLADH coupled assay due to the high UV absorbance of these compounds at 250 nm. The results of this survey are summarized pictorially in Chart 1. It becomes readily apparent that MOI is unique in its strong affinity for MNL, as several structural analogs have K_is up to 40-fold greater than that for MOI. The indole or indoline ring contributes very little to strong binding recognition as the dissociation constants for these compounds are near 1 mM. It was anticipated that the amide functional group of the lactam ring of MOI, possibly in combination with a nonpolar substituent at position 3, might be key contributors in binding interactions. Oxindole, 3-methyloxindole, and skatole were suitable models for these characteristics, and although these compounds are better inhibitors than the unsubstituted indole derivatives, the dissociation constants are still 20–30-fold higher than that for MOI. These results indicate that indoles are modest competitive inhibitors of MNL, with dissociation constants comparable to the K_m of the natural substrate, D,L-mandelonitrile. However, the structural features examined revealed very little insofar as explaining the high affinity of MNL for MOI, suggesting that the chemical characteristics and tautomeric structures of MOI must be evaluated.

Modification of Cysteine Residues and Enzyme Reactivation. Methyleneoxindole is known to react with a variety of low molecular weight thiols, presumably through conjugate addition reactions at the exocyclic double bond (Hinman et al., 1961, 1964b; Still et al., 1965). As MNL is readily inactivated by a variety of thiol-specific reagents (Jaenicke & Preun, 1984; Brush and Yang, unpublished data), it became essential to determine if thiol residues on MNL were being alkylated by MOI. In the presence of 6 M guanidine, 3.1 ± 0.1 cysteine residues were detected in the untreated enzyme using Ellman's reagent, DTNB (Ellman, 1959); however, a sample of MNL inactivated by MOI indicated that two thiol residues had been modified. Furthermore, under the conditions of the DTNB assay (pH 7.3), the putative MOI–cysteine covalent adducts appear to be labile, as the number of free cysteine residues detected by DTNB increased with time. One minute after the addition of DTNB to the MOI-modified protein, 1.1 ± 0.2 thiols were titrated, but after 30 min the maximum number of 3.1 ± 0.1 thiols was observed.

These results indicate that the MNL–MOI adduct is unstable under the conditions of the DTNB titration (0.1 M phosphate buffer, pH 7.3). This possibility was examined further using the *N*-acetylcysteine–MOI adduct. Incubation of a 12.5 μM solution of the adduct with a 20-fold excess of DTNB in 0.1 M phosphate buffer, pH 7.3, resulted in the recovery of essentially all *N*-acetylcysteine (12.2 μM) within 40 min. These results prompted us to carefully examine the stability of the MNL–MOI adduct under nondenaturing conditions by testing for recovery of enzymatic activity. Samples of MNL were incubated with MOI until less than 10% of the original activity remained. Immediately following spin dialysis into fresh citrate buffer, pH 5.5, no activity recovery was observed, consistent with covalent modification of protein amino acid residues. However, over a 5-h time period at room temperature the retentate slowly recovered approximately 30% of the original activity, as compared to a control treated in an identical fashion but in the absence of MOI.

These results suggest the formation of a labile covalent adduct between MOI and amino acid functional group(s) of MNL, possibly with one or two cysteine residues. To probe for the formation of an unstable covalent adduct that is sensitive to dilution, samples of MNL were inactivated to ca. 10% of their original activity with 0.1 mM MOI at pH 5.5 and immediately treated with 1 mM 2-mercaptoethanol or DTT, and the effect on MNL activity recovery was monitored as a function of time. Surprisingly, a time-dependent reactivation reaction was observed, with the results for DTT illustrated in Figure 4. The initial velocity data for enzyme reactivation were fit to

$$V_{(t)} = V_0 + V_{max}(1 - e^{-k_r t}) \quad (4)$$

which describes the pseudo-first-order recovery of enzymatic activity, where V_0 is the initial velocity of MOI-modified MNL, V_{max} is the maximum velocity recovered, and k_r is the apparent pseudo-first-order rate constant for reactivation (Todd & Hausinger, 1991). A good fit to eq 4 for both DTT and β-mercaptoethanol was observed (data not shown), with calculated k_r values of 6.8×10^{-3} and 6.7×10^{-3} min^{-1}, respectively. The similarity of the reactivation rate constants for these two thiols suggests that thiol structure has little

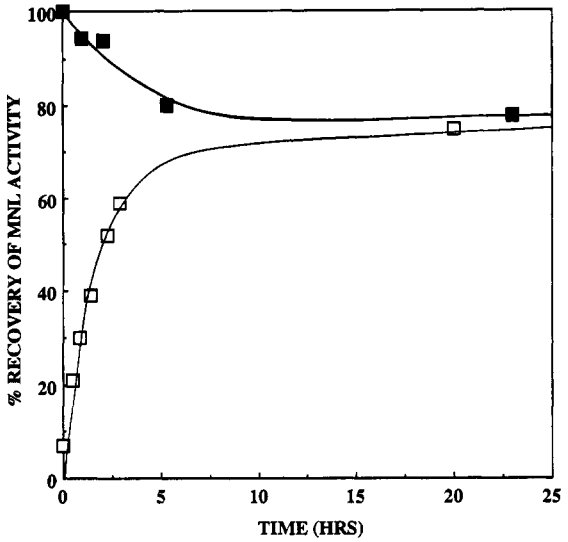


FIGURE 4: Recovery of activity from MOI-modified MNL following treatment with 1 mM DTT (□). A control was treated similarly but in the absence of MOI (■).

effect on activity recovery, which may be limited by the breakdown of the MNL–MOI covalent adduct. Synthesis of radiolabeled MOI is currently in progress in order to accurately evaluate the inactivation stoichiometry and to determine the chemical reactivity of the putative protein-bound adduct. This information will be critical in our efforts to isolate and sequence MOI-modified peptides and identify the reactive amino acid residues.

pH Dependence on the Inactivation of MNL by MOI. The available data suggest that MOI is an effective active-site-directed inhibitor of MNL, which forms an unstable covalent adduct through the modification of protein thiol residues. The inactivation reactions were all conducted at pH 5.5, which is the optimum pH of the MNL-catalyzed reaction (Jorns, 1980). At this pH, and in the presence of micromolar concentrations of MOI, MNL is rapidly inactivated, with half-lives on the order of minutes (Figure 2). The pH of the inactivation reaction is significantly lower than the pK_a of thiols in solution (9.0–9.6), such that at pH 5.5 greater than 99% of the thiol would be in the protonated form. Protonated thiols have been shown to be over 5×10^{10}-fold less reactive than the thiolate anion in Michael addition reactions to *N*-ethylmaleimide (Bednar, 1990). In the inactivation reaction, nucleophilic addition requires the thiolate ion as the reactive species; hence the pK_a of the thiol must be low enough to produce the thiolate anion at the pH of the reaction. It became desirable to evaluate the effect of pH on the inactivation reaction between MOI and MNL, in order to better evaluate the pK_a of reactive active-site functional groups which may be modified by MOI.

Detailed studies on the effect of pH on the normal reaction catalyzed by the oxynitrilases are not possible given the rapid nonenzymatic decomposition of cyanohydrins at pH values greater than 6.5. Hence, the pH dependence on the rate of inactivation by active-site-directed irreversible inhibitors can be a useful way of characterizing such compounds and possibly learning more about the reactive active-site functional groups involved in normal catalysis of cyanohydrin substrates. However, appropriate caution must be exercised in order to avoid overinterpretation of kinetic pK_a values derived from inactivation rate data or the assignment of a kinetic pK_a to the ionization of a specific amino acid functional group (Cleland, 1982; Knowles, 1976). Figure 5 illustrates the effect of pH on the second-order rate constant for the inactivation

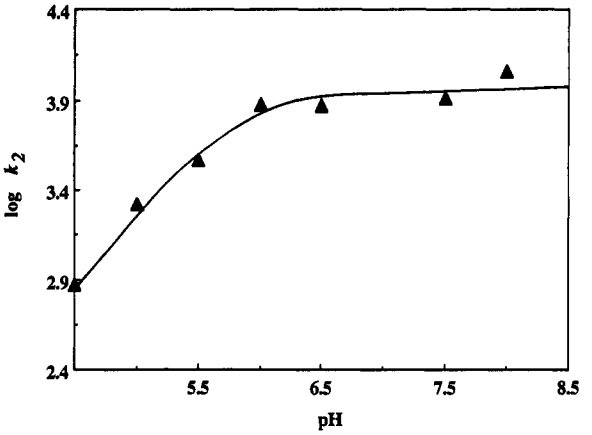


FIGURE 5: pH dependence on the inactivation of MNL by MOI. The log of the apparent second-order rate constant (M^{-1} min^{-1}) is plotted as a function of pH for the inactivation reaction.

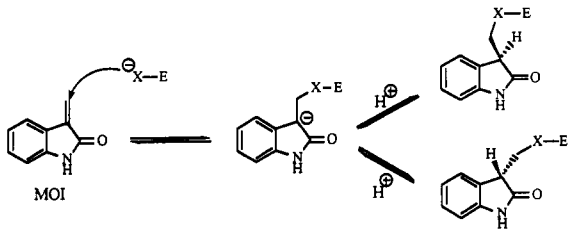
reaction, k_2. Inactivation of MNL by MOI was found to follow pseudo-first-order kinetics between pH 4.5 and 8.0. At pH values greater than 8.0, first-order kinetics were not observed due to MOI instability. Values for k_2 were fit to eq 5, where K_a is the acid dissociation constant for the amino acid residue involved in the inactivation reaction.

$$\log k_2 = \log \{c/(1 + [H^+]/K_a)\} \quad (5)$$

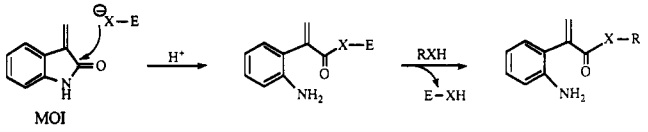
A good fit to eq 5 was obtained, which describes a pH–rate profile that goes from a slope of 1 below the apparent pK_a to a slope of 0 above the apparent pK_a of the functional group being modified. The pH profile is not complicated by the ionization of MOI in this pH range. Although data on the pK_a for ionization of the NH group for oxindole derivatives are not available, a reasonable estimate may be made from substituted indoles. A pK_a of 17 has been determined for NH ionization of indole, while the vinylogous indoleamide, 3-formylindole, has a pK_a of 12.4 (Yagil, 1967). So over the pH range employed, inhibitor ionization is expected to make an insignificant contribution to the pH–rate profile, and the data in Figure 5 suggest that inactivation of MNL by MOI depends on the ionization of a single functional group with an apparent pK_a value of 5.6 ± 0.03. These data will be useful in characterizing the pH effect on the inactivation of MNL by active-site-directed reagents and mechanism-based inactivators, as well as active-site mutagenesis studies.

Possible Mechanisms for the Inactivation of MNL by MOI. 3-Methyleneoxindole has been suggested to be a growth regulator in both plants and microorganisms (Fukuyama & Moyed, 1964). These effects are presumably mediated by the ability of MOI to covalently modify proteins and enzymes, although neither the site of modification, structure of the protein-bound adducts, or mechanisms have been elucidated (Still et al., 1965). The addition of low molecular weight thiols or bisulfite to aqueous solutions of MOI results in a change of its characteristic absorption spectrum to one that is very similar to that of 3-methyloxindole, with the formation of an addition compound indicated by the inability to extract free MOI from aqueous solutions with diethyl ether (Hinman et al., 1961; Still et al., 1965). Hinman and Bauman (1964b) isolated the addition compound formed between thiophenol and MOI and identified it as 3-(phenylthiomethyl)oxindole. These results suggest that the adduct formed in the reaction of MOI with protein cysteine residues is a thioether formed *via* Michael addition reaction of the nucleophile at the exocyclic double bond (Scheme 4). The benzylic C-3 carbanion is

Scheme 4



Scheme 5



stabilized by resonance through the aromatic ring and with the neighboring lactam carbonyl, with protonation occurring from either face of the oxindole ring, producing diasteriomeric adducts.

The thiol-addition compounds are reported to be unstable, reverting back to MOI and the free thiol, especially when subjected to alkaline conditions (Hinman et al., 1964b; Still et al., 1965). Elimination of the thiol leaving group requires deprotonation at C-3, re-forming the resonance-stabilized carbanion. The pK_a of the carbon-3 proton in an oxindole Michael adduct is not known, although it has been reported that the carbon-3 proton of oxindoyl-L-alanine completely exchanges in acidic ^2H_2O (Ohno et al., 1974). The reversibility of the Michael adduct is consistent with our observation of partial activity recovery following spin dialysis of MOI-inactivated enzyme. Furthermore, nearly stoichiometric enzyme reactivation occurs following the removal of excess MOI by the alkylation of added DTT or 2-mercaptoethanol to the inactive enzyme (Figure 4). It is also possible that the added nucleophile could facilitate elimination of the adduct by acting as a base to remove the C-3 proton or as a nucleophile *via* an S_N2 displacement. At pH 5.5 neither of these possibilities is attractive given the low concentration (<1%) of the reactive thiolate ion under these conditions.

An alternative, though less appealing, mechanism which is also consistent with the inactivation and reactivation reactions is illustrated in Scheme 5. Attack of the protein nucleophile could occur at the carbonyl carbon, resulting in opening of the oxindole ring and formation of an α,β-unsaturated acyl–enzyme adduct. The chemistry of oxindoles is reportedly similar to that of simple lactams, and oxindole ring opening has been shown to occur intramolecularly by nucleophilic groups in the C-3 position or by intermolecular attack of acetate ion (Wenkert et al., 1958). Reactivation of MOI-modified MNL would then result from hydrolysis of the acyl–enzyme or by nucleophilic acyl substitution.

The instability of the MOI–protein adducts has currently precluded our attempts to unambiguously identify the modified amino acid residue. The synthesis of radiolabeled MOI from [^{14}C]IAA and detailed studies on the chemical stability of the adduct are in progress. However, we have shown that MNL inactivation by MOI proceeds with the loss of two thiol residues, as determined using Ellman's reagent. Furthermore, MOI has been reported to lose its characteristic UV absorption spectrum in the presence of glutathione (Still et al., 1965). Hence, an active-site cysteine residue is a reasonable candidate for the enzyme nucleophile, and the estimated apparent pK_a of 5.6 (Figure 5) would assure that a significant concentration of the thiolate ion will exist at pH 5.5, the optimum pH for the MNL-catalyzed reaction. As the pK_a of the thiol group in cysteine is between 9 and 9.5, the active-site environment must help lower the pK_a of the reactive thiol(s). The low pK_as of active-site thiol groups in papain (pK_a of 3.2) (Lewis et al., 1976) and glutathione reductase (pK_a of 4.8) (Arscott et al., 1981) have been attributed to a nonpolar active-site environment and the presence of a functional thiol–base ion pair. This is an attractive hypothesis for MNL since the thiolate ion could perform important functions as an active-site base in the processing of normal substrates. We have suggested that the putative thiolate ion may function in normal catalysis as a base to deprotonate the cyanohydrin substrate, forming the oxyanion which facilitates expulsion of the cyanide leaving group (Brush and Yang, submitted for publication). Our continuing efforts are directed at testing these hypotheses.

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