Interaction of L-Canaline with Ornithine Aminotransferase of the Tobacco Hornworm, Munduca sexta (Sphingidae)*

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Ornithine aminotransferase (L-ornithine:2-oxo-acid aminotransferase (EC 2.6.1.13)) has been purified to homogeneity from last instar larvae of the tobacco hornworm, Munduca sexta (Sphingidae). This enzyme is a 144,000-Da tetramer constructed from 36,000-Da protomeric units. It has a high aspartate/asparagine and glutamate/glutamine content and 2 cysteine residues/monomer. All 8 cysteine residues can react with N-ethylmaleimide to inactivate the enzyme. Maintenance of the enzyme in the presence of 2-mercaptoethanol and dithiothreitol maximizes enzymatic activity and improves storage conditions, presumably by protecting these sulfhydryl groups. The apparent \( K_m \) values for L-ornithine and 2-oxoglutaric acid are 2.3 and 3.2 \( \mu M \), respectively. The turnover number is \( 2.0 \pm 0.1 \) mol min\(^{-1}\\mu M\)^\(-1\)

L-Canaline (L-2-amino-4-(aminooxy)butyric acid) is a potent ornithine aminotransferase inhibitor. Reaction of the enzyme with L-[U-\(^{14}C\)]canaline produces an enzyme-bound, covalently linked, radiolabeled canaline-pyridoxal phosphate oxime. The L-[U-\(^{14}C\)]canaline-pyridoxal phosphate oxime has been isolated from canaline-treated enzyme. Dialysis of canaline-inactivated ornithine aminotransferase against free pyridoxal phosphate slowly reactivates the enzyme as the oxime is replaced by pyridoxal phosphate. Analysis of L-[U-\(^{14}C\)]canaline binding to ornithine aminotransferase reveals the presence of 4 mol of pyridoxal phosphate/mol of enzyme.

Larvae of insects such as the tobacco hornworm, Munduca sexta (Sphingidae), generate L-glutamic acid by several mechanisms including: glutamic dehydrogenase (EC 1.4.1.2)-mediated reductive amination of 2-oxo-acids, from proline through pyrroline-5-carboxylic acid, degradation of larval protein, and an aminotransferase reaction in which 2-oxoglutarate serves as a prime source of carbon skeleton (1).

Kinetic analysis of glutamic dehydrogenase from larval M. sexta reveals that the \( K_m \) for ammonia exceeds 400 \( \mu M \), a value well above its physiological concentration (2). Glutamine is not a nitrogen donor to this insect dehydrogenase. Thus, this reaction pathway probably contributes little to glutamate biosynthesis. Glutamic acid production from proline is important in such tissues as adult flight muscles where proline consumption is coupled to ATP formation (3). In the larva, however, glutamate formation from proline is limited since the latter amino acid is not a major component of M. sexta hemolymph (4). Analysis of M. sexta fails to disclose significant larval glutaminase activity, thereby precluding such deamination as an important source of glutamic acid. Glutamic acid can be supplied by catabolism of hemolymph storage proteins, but Kramer et al. (5) found that the major function of these macromolecules is to provide aromatic amino acids. Larval aminotransferases other than ornithine aminotransferase (L-ornithine:2-oxo-acid aminotransferase (EC 2.6.1.13)) (OAT)\(^1\) that are able to use 2-oxoglutarate can provide glutamic acid; but such larval pathways have not been evaluated. Thus, the reaction fostered by OAT, while not the sole reaction, is nevertheless an important means for glutamic acid formation in insects such as M. sexta.

Insects produce ample ornithine through arginase-directed hydrolysis of L-arginine; this enzyme is distributed widely among these arthropods (6). Since insects lack a functional Krebs-Henseleit ornithine/urea cycle (7, 8), ornithine is not lost through arginine biosynthesis. In spite of the importance of OAT, this protein has not been purified previously from an insect.

L-Canaline, the L-2-amino-4-(aminooxy)butyric acid structural analog of L-ornithine, is a potent antimetabolite of higher plants (9). Indirect evidence, such as the shift in absorption spectrum that occurs when canaline reacts with an appropriate protein, suggests that this nonprotein amino acid interacts with the pyridoxal phosphate moiety of vitamin B\(_6\)-containing proteins to form a stable, covalently linked complex (10, 11).

The experiments detailed in this study were designed to purify and characterize OAT and to analyze its interaction with L-canaline.

EXPERIMENTAL PROCEDURES

Materials

Terminal instar tobacco hornworm larvae were reared as described previously (12). Unless otherwise indicated, all biochemical reagents were purchased from Sigma.

Substrate Preparation

L-Canaline was prepared from its dipicrate salt obtained from L-canavanine (13). L-Canavanine was isolated from acetone-defatted jack bean seeds (Canavalia ensiformis) and purified by repetitive recrystallization (14).

L-[U-\(^{14}C\)]Canaline was synthesised from commercially prepared L-[U-\(^{14}C\)]homoserine (Amersham Corp.; 1.48 GBq/mmol). The radioactivity is important in such tissues as adult flight muscles where proline consumption is coupled to ATP formation (3). In the larva, however, glutamate formation from proline is limited since the latter amino acid is not a major component of M. sexta hemolymph (4). Analysis of M. sexta fails to disclose significant larval glutaminase activity, thereby precluding such deamination as an important source of glutamic acid. Glutamic acid can be supplied by catabolism of hemolymph storage proteins, but Kramer et al. (5) found that the major function of these macromolecules is to provide aromatic amino acids. Larval aminotransferases other than ornithine aminotransferase (L-ornithine:2-oxo-acid aminotransferase (EC 2.6.1.13)) (OAT)\(^1\) that are able to use 2-oxoglutarate can provide glutamic acid; but such larval pathways have not been evaluated. Thus, the reaction fostered by OAT, while not the sole reaction, is nevertheless an important means for glutamic acid formation in insects such as M. sexta.

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chemical synthesis involved the sequential formation of N-benzyl-
N-carboxyhomoserine, N-benzyl-N-carboxyhomoserine lactone,
and O-benzyl-N-benzyl-N-carboxyhomoserine. Tosylation yielded
the trifunctional derivative benzyl L-2-(carbobenzyloxy)amino-4-[p-
tosylsulfonyl]oxy]butyrate. The trifunctional derivative was treated
with benzoylhydroxamic acid to introduce the aminooxy function and
then deprotected by refluxing with 15% (w/v) ethanolic HCl followed
by 3 N HCl (16). The free amino acid was prepared by treatment with
triethylamine (16).

Methods

Enzyme Assay

OAT activity was determined by measuring the formation of L-
glutamic acid semialdehyde from L-ornithine; this semialdehyde cy-
clicized spontaneously to yield 6 pyrroline-5-carboxylic acid. The assay
mixture (0.5 ml) consisted of 100 mM sodium Tricine containing 0.5
mM EDTA, pH 8.0, 30 mM L-ornithine, 40 mM 2-oxoglutarate, 1 mM
pyridoxal phosphate, 0.4 mM dithiothreitol, 0.4 mM 2-mercaptoeth-
ol, and no more than 5 x 10^{-3} unit of OAT (in 0 ml of standard
buffer). The standard buffer consisted of 250 mM sodium Tricine, pH
8.0, 1.25 mM EDTA, 0.02 mM pyridoxal phosphate, 1 mM dithiothre-
itol, and 1 mM 2-mercaptoethanol. All assays were conducted in
triplicate at 37 °C for 45 min.

Enzyme assays were terminated by addition of 0.1 ml of 30% (w/ v)
trichloroacetic acid. 6-Pyruvyl-5-carboxylic acid was determined by
reacting the reaction mixture with 0.5 ml of a freshly prepared
solution of o-aminobenzoic acid (5 mg/ml) in absolute ethanol. 6-
Pyruvyl-5-carboxylic acid reacted with this reagent to form a deep
yellow dihydroxybenzaldehyde derivative (17). Controls consisted of
appropriate reaction mixtures to which trichloroacetic acid was added
prior to OAT. One unit of OAT catalyzed formation of 1 μmol of 6-pyruvyl-5-carboxylic acid min^{-1} under the described conditions.

Protein Assay

Protein was determined in crude preparations by the method of
Lowry et al. (18) employing bovine serum albumin as the standard.
The absorbance of purified OAT was determined with the exhaust-
ively dialyzed enzyme that was dried to constant weight in vacuo at
100 °C. A 0.1% (w/v) solution of OAT had an E_{280} of 0.96.

Polyacrylamide Gel Electrophoresis

OAT was analyzed by polyacrylamide electrophoresis under non-
denaturing conditions by the method of Davis (19). The protein, treated with sodium dodecyl sulfate under reduced conditions for 3
min at 100 °C, was also analyzed by the method of Laemmli (20). All
gels were stained with 0.05% (w/v) Coomassie Blue. Commercially
prepared proteins (ranging from 14,000 to 181,000 Da) served as the
standards (Sigma SDS-6 and SDS-7 standards).

Amino Acid Analysis

Samples of OAT (100 μg) were hydrolyzed in 6 N HCl with 0.02%
(w/v) 2-mercaptoethanol and 0.01% (v/v) phenol for 24–72 h at 100 °C
in vacuo. The amino acid composition of the hydrolysate was estab-
lished by ion-exchange chromatography using a Dionex D-300 auto-
inated amino acid analyzer equipped with a lithium citrate physiolog-
ical buffer system and ninhydrin detection. Protein amino acids were
standardized with Fierce amino acid standard H. Trypsinogen and
half-eyestripe were determined as previously described (21).

Kinetic Constants

The kinetic constants for canaline-dependent oxime formation
were determined by measuring fluorescent intensity by the method
of Beeler and Churchich (11). Fluorescence was measured with a
Perkin-Elmer Model 660-105 recording fluorometer. slit width was 5 nm, and sensitivity was 0.3.

Immunization and Preparation of Rabbit Anti-OAT Antibodies

Homogeneous OAT (1 mg in 1 ml of 0.9% (w/v) NaCl) was
emulsified with an equal volume of Freund's complete adjuvant
(Difco) and administered by multiple subcutaneous dorsal injections
into a rabbit. Comparable booster injections with incomplete adjuvant
were given after 2 and 4 weeks. After 6 weeks, blood was taken from
an ear vein and clotted, and the serum was collected. The IgG fraction
was obtained by using liquid-saturated ammonium sulfate to the
serum until a final concentration of 40% (v/v) was achieved. Precip-
itated proteins were collected by centrifugation, dissolved in 50 mM
Tris-HCl, pH 8.0, and reprecipitated with ammonium sulfate as
described above. The IgG fraction was dissolved in 3 ml of 25 mM
Tris-HCl, pH 8.0, containing 125 mM NaCl and dialyzed exhaustively
against this saline buffer. These materials, representing the standard
IgG preparation, were stored at -60 °C.

Antigen-Antibody Interaction

The effect of rabbit anti-OAT antibodies on the activity of the
enzyme was determined by incubating the indicated amount of IgG
from commercial sources with 3.5 μg of OAT in standard buffer at 30 °C
for 30 min prior to evaluating enzymatic activity. Control samples did
not contain IgG fraction materials.

Kinetic Analysis

Apparent K_m values were taken from Lineweaver-Burk plots de-
termined by regression analysis. Lines having an r values less than
0.990 were discarded. Turnover number was calculated from V_max
and is described fully elsewhere (22, 23).

L-[U-^{14}C]Canaline Binding Studies

Reaction of [^{14}C]canaline with OAT to form the radiolabeled

canaline-pyridoxal phosphate oxime was established by reacting OAT
with [^{14}C]canaline as described above. Unreacted radiolabeled cana-
line and the free oxime were removed by passing the remaining
reaction mixture (0.75 ml) over a 10 x 250-mm column of Sephadex
G-10. To ensure the complete removal of L-[U-^{14}C]canaline and the
radiolabeled oxime, the OAT-bearing fractions were lyophilized, and
the protein was taken up in 50 mM Tricine, pH 8.0, and treated with
2 volumes of liquid-saturated ammonium sulfate. After standing at
6 °C for 30 min, the turbid solution was centrifuged at 12,000 x g for
12 min; this procedure was repeated three times. Radiolabeled OAT
was then dialyzed for 72 h against ultrapure water (Millipore Nona-
pure System). The radiolabeled oxime was concentrated from the
dialysate by rotary evaporation in vacuo. The [^{14}C]canaline-pyridoxal
phosphate oxime was isolated by automated amino acid analysis as
described above. Bovine serum albumin which was processed as
described above served as the control; the dialysate was free of [^{14}C].

Canaline Inhibition Studies

The ability of canaline to inhibit OAT was determined by reacting
7.2 μg of OAT in 0.5 ml of standard buffer with the indicated amount
of canaline at 31 °C (final volume, 1 ml). At the indicated time, 0.1
ml of the reaction mixture was assayed for enzymatic activity. The
time reaction mixture contained deionized water instead of cana-
line. The control reaction mixture contained 144,000 Da.

Purification of M. sexta OAT

All extraction procedures were conducted at 4 °C using either
sodium phosphate buffer, pH 8.0, containing 0.25 mM EDTA, 1 μM
2-mercaptoethanol, 1 mM dithiothreitol, and 0.02 mM pyridoxal
phosphate (standard phosphate buffer) or 50 mM sodium Tricine, pH 8.0,
0.25 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM dithiothreitol, and
0.02 mM pyridoxal phosphate (standard Tricine buffer). Unless oth-
wise indicated, centrifugation was conducted at 15,000 x g for 15
min. Standard dialysis buffer consisted of 50 mM sodium Tricine, pH
8.0, 0.25 mM EDTA, 1 mM dithiothreitol, and 0.02 mM pyridoxal
phosphate (standard buffer). DH 8.0, containing 0.25 mM EDTA
and 0.02 mM pyridoxal phosphate (standard buffer).
8.0, 0.25 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM dithiothreitol, and 0.02 mM pyridoxal phosphate.

Preparation of Larval Extract—Frozen M. sexta larvae (200 g; wandering larval stage) were homogenized in a Waring Blender for 1 min at full power with 170 ml of 200 mM standard phosphate buffer containing 0.025% (w/v) phenylthiourea. Phenylthiourea retarded tyrosinase activity and effectively prevented discoloration of the larval extract. Twenty min later, the homogenate was centrifuged, and floating debris was removed from the supernatant solution by filtering through 100% polyester aquarium filter floss.

Ammonium Sulfate Precipitation—The filtered homogenate was brought to 20% saturation with solid enzyme-grade ammonium sulfate, stirred for 30 min, and then allowed to stand for 60 min prior to centrifugation. The supernatant solution was filtered through polyester floss, taken to 40% saturation with solid ammonium sulfate as described above, and centrifuged. The supernatant solution was treated with ammonium sulfate to a final concentration of 55% (w/v). One h later, the turbid solution was centrifuged, and the green pellet was dissolved in 5 mM sodium phosphate buffer and taken to a final volume of 50 ml.

Ammonium sulfate was removed by dialysis against 25 volumes of buffer; the standard dialysis buffer was changed once during 20 h. Centrifugation was used to remove protein that precipitated during dialysis.

Heat Treatment—The dialyzed enzyme (8-ml aliquots) was taken to 60°C using a 90°C water bath. At that time, it was transferred to a 60°C water bath and maintained at this temperature for 4 min. The turbid solution was clarified by centrifugation at 40,000 x g for 1 h.

DEAE-cellulose Chromatography—The supernatant solution from the heat treatment was applied to a 20 × 250-mm column of Whatman DEAE-cellulose (DE32) equilibrated with 5 mM sodium phosphate buffer, pH 8.0, without additional components. After applying the sample, the column was washed with 200 ml of 5 mM standard phosphate buffer. The enzyme was eluted with 140 ml of 50 mM standard phosphate buffer. The effluent, at a flow rate of 0.35 ml min⁻¹, was collected as 4.8-ml fractions. All fractions possessing a specific activity of at least 0.50 were pooled (Fig. 1).

Sephadex G-200 Chromatography—The pooled effluent (~40 ml) was concentrated to 2 ml with an Amicon ultrafiltration cell fitted with a PM-10 membrane. One-half was passed over a 10 × 500-mm column of Sephadex G-200 equilibrated with standard Tricine buffer. Fractions (2 ml) were collected at 15-min intervals; fractions having a specific activity of at least 1.6 were pooled (Table I).

OAT exhibited a sharp loss in activity at 1°C, especially in the absence of free pyridoxal phosphate and mercaptans. Purified OAT could be stored in standard Tricine buffer for several weeks at -60°C. Long-term OAT storage was best as a pellet under liquid-saturated ammonium sulfate at -60°C. Prior to use, the enzyme was dialyzed against standard Tricine buffer.

Criteria of Purity—The immunological homogeneity of purified OAT was evaluated by the Ouchterlony double-diffusion technique (24), which revealed a single precipitation line. Antibody-directed inhibition of OAT constitutes Fig. 2. Electrophoretic evidence of purity is presented below.

RESULTS

Characterization of M. sexta OAT:Mass—The molecular mass of the OAT subunit was obtained by SDS-polyacrylamide gel electrophoresis utilizing proteins of known mass as the standard. This procedure yielded a single band with an apparent minimum subunit molecular mass of 36,000 Da.

The molecular mass of M. sexta OAT was determined to be 148,000 ± 5,000 Da by polyacrylamide gel electrophoresis under nondenaturing conditions. Molecular mass evaluation by Sephadex G-200 chromatography yielded a value of 152,000 ± 6,000 Da. It appears that this insect OAT exists as a tetramer composed of four 36,000-Da subunits.

Amino Acid Analysis—Automated amino acid analysis of OAT disclosed its high glutamate/glutamine content. Aspartate/asparagine are the next most abundant amino acids. This protein also has numerous basic and hydrophobic amino acids. Automated amino acid analysis revealed 8 cysteine residues,
TABLE I
Purification of ornithine aminotransferase from larval M. sexta
Experimental details are provided in the text.

| Fraction            | Total activity | Protein | Specific activity | Recovery |
|---------------------|----------------|---------|------------------|----------|
|                     | units          | mg      | units/mg         | %        |
| Larval extract      | 230            | 15,540  | 0.015            | 50       |
| Ammonium sulfate (40-55%) | 115          | 3,590   | 0.032            | 50       |
| Heat treatment      | 55             | 625     | 0.88             | 24       |
| DEAE-cellulose      | 17             | 18      | 0.94             | 7        |
| Sephadex G-200      | 4.0            | 2.5     | 1.60             | 1.7      |

Fig. 2. Antigen-antibody interaction. OAT was treated with rabbit anti-OAT antibodies as described under "Antigen-Antibody Interaction."

Fig. 3. Apparent $K_m$ values. Apparent $K_m$ values were determined as described in text.

Fig. 4. OAT-canaline interaction. Canaline-dependent inactivation of OAT was determined as described under "Canaline Inhibition Studies." O, control, △, ○, ●, and ▲, 10$^{-8}$, 10$^{-7}$, 10$^{-6}$, and 10$^{-5}$ M canaline, respectively.

A value in accord with that obtained by Ellman titration (Table II).

Kinetic Parameters—Kinetic analysis of OAT indicated that the apparent $K_m$ values for L-ornithine and 2-oxoglutaric acid are 2.3 x 10$^{-3}$ and 3.2 x 10$^{-3}$ M, respectively (Fig. 3). The highest specific activity OAT gave a turnover number of 2.0 ± 0.1 µmol/min/µmol OAT.

Pyridoxal Phosphate Content—The pyridoxal phosphate content of OAT was determined by reaction with L-[U-14C] canaline and evaluation of the radiolabeled protein as described. These determinations established that M. sexta OAT contained 4 mol of pyridoxal phosphate/mol of enzyme.

OAT-Canaline Interaction—Treatment of OAT with canaline caused a rapid and precipitous loss of activity (Fig. 4). At 10$^{-6}$ M canaline, for example, nearly three-fourths of the enzymatic activity was lost after a 5-min exposure at 21 °C. Treatment with as little as 10$^{-7}$ M canaline manifested a significant reduction in activity. This is an interesting observation since when OAT was exposed to 10$^{-6}$ M canaline, there was a 1000-fold excess of free pyridoxal phosphate relative to canaline in the reaction mixture (pyridoxal phosphate is required to protect the enzyme). Other workers (11, 25) have noted the ability of canaline to react preferentially with the pyridoxal phosphate moiety of rat liver OAT in the presence...
of a large excess of free pyridoxal phosphate. Evaluation of the second order rate constant for the formation of the canaline-pyridoxal phosphate oxime disclosed that the reaction rate was more than 16 times greater for OAT in the complete buffer system as compared to free pyridoxal phosphate (Table III). The rate constant fell from 4380 M⁻¹ min⁻¹ for the complete buffer system to 2895 M⁻¹ min⁻¹ when both pyridoxal phosphate and mercaptans were absent. In the presence of buffered mercaptans, the rate constant was 3215 M⁻¹ min⁻¹ as compared to 2895 M⁻¹ min⁻¹ for buffered pyridoxal phosphate (Table III).

Formation of Canaline-Pyridoxal Phosphate Oxime—To establish that canaline reacted with the pyridoxal phosphate moiety of OAT, the enzyme was treated with [¹⁴C]canaline, and the radiolabeled canaline-pyridoxal phosphate oxime was released by dialysis against deionized water. The radiolabeled oxime was analyzed by automated amino acid analysis as described. The [¹⁴C]canaline-pyridoxal phosphate oxime possessed a column retention time of 87 min. The retention time for the chemically prepared canaline-pyridoxal phosphate oxime is also 87 min. Analysis for [¹⁴C]in the spent ninhydrin, following the method of Rosenthal (26), indicated that virtually all of the dialysate [¹⁴C]eluted with the canaline-pyridoxal phosphate oxime. Thus, it was possible to isolate the [¹⁴C]canaline-pyridoxal phosphate oxime created by treating OAT with [¹⁴C]canaline.

The canaline-pyridoxal phosphate complex was dissolved in H₂O and analyzed by NMR using tetramethylsilane as an internal standard. Such analysis disclosed the anticipated downfield shift of the α, β, and γ canaline protons (δ = 0.12, 0.2, and 0.7 ppm, respectively). The resonance signal at 8.9 ppm, due to the methine proton of the Schiff-base linkage between canaline and pyridoxal phosphate, was readily discernible.

Further identification of the canaline-pyridoxal phosphate oxime was achieved by discovery of its appreciable fluorescence after excitation at 380 nm (Fig. 5). A 1 × 10⁻⁴ M solution of canaline-pyridoxal phosphate, excited at 380 nm at pH 7.5, had a fluorescent intensity of 20.4 at 460 nm. The extreme sensitivity of this assay permitted comparative fluorescent analysis of canaline-pyridoxal phosphate oxime, obtained from [¹⁴C]canaline-treated OAT, with the chemically prepared material analyzed in Fig. 5. These analyses revealed that the emission pattern of the OAT-derived radiolabeled canaline-pyridoxal phosphate complex was identical to that of the chemically prepared material. Under these experimental conditions, neither canaline nor pyridoxal phosphate possessed detectable fluorescence over the range of 400–600 nm.

Sulfhydryl Inactivation—The essentiality of the sulfhydryl groups of M. sexta OAT was demonstrated by reaction with N-ethylmaleimide. As shown in Fig. 6, this reagent elicited a concentration-dependent inactivation of OAT. Amino acid analysis and titration of OAT with 5,5'-dithiobis-(2-nitrobenzoic acid) following the method of Ellman (27) indicated that the tetramer contained 8 cysteines, presumably 2/subunit. Treatment of OAT with 6 M guanidine, prior to reaction with the Ellman reagent, failed to increase the cysteine titer. Thus, all of the cysteines were exposed and readily available to this reagent.

**DISCUSSION**

Ornithine aminotransferase isolated from larval M. sexta exists in a single discernible form: a 144,000-Da tetramer composed of four 36,000-Da monomers. This contrasts with rat liver OAT as studied by Sanada et al. (28). The mammalian
enzyme that they studied is formed from two different apoproteins, each containing a 45,000 ± 2,000-Da protomer. The apoproteins possess a mass of 177,000 ± 7,700 or 105,000 ± 6,300 Da (28). Earlier, Peraino et al. (29) crystallized rat liver OAT and determined its mass by equilibrium ultracentrifugation to be 132,000 Da. They believed that like the insect protein, it is a tetramer, but built of 32,000-Da prometric units. These workers noted the disparate literature OAT mass values and suggested that the reported discrepancies may result from the dependence of the calculated mass on protein concentration.

There is much homology as well as significant differences in the primary structure of M. sexta OAT and those of the rat liver and kidney proteins. Glutamic acid and leucine are the most abundant residues in the mammalian enzymes, whereas histidine, methionine, and tryptophan are the least common (30). The cysteine content of liver and kidney OATS is 16 and 14, respectively. Whereas glutamic acid is also the most abundant residue in the insect protein, the leucine level is low and is exceeded by the isoleucine content. Histidine and tryptophan are limited, but the methionine content is higher, exceeding that of serine and phenylalanine. The thiol group content is about half of that for the mammalian enzymes (Table II).

[14C]Canaline provides an effective tool for determining the pyridoxal phosphate content of this insect enzyme. 14C Labeled canaline reacts stoichiometrically with the pyridoxal phosphate of OAT to form a stable, radiolabeled oxime that can be removed by simple dialysis. 14CCanaline-protein binding analysis may provide a sensitive and convenient radiometric assay for determining the pyridoxal phosphate content of vitamin B6-containing proteins.

Reaction of canaline with OAT to form a canaline-pyridoxal phosphate oxime rapidly inactivates OAT. This oxime can be removed by dialysis to create an apoenzyme that can react with free pyridoxal phosphate to regenerate a catalytically competent macromolecule (Table IV). These experiments provide the first direct chemical evidence that canaline inactivates pyridoxal phosphate-containing proteins by forming an oxime with the cofactor that blocks its catalytic activity.

Analysis of the rate constant for the reaction of canaline with OAT suggests that when the enzyme is protected by pyridoxal phosphate and mercaptans, thereby most effectively stabilizing the native conformation, it is most reactive with canaline (Table III). Interestingly, 2-mercaptooctanol- and dithiobiotreitol-protected OATS in buffer yield a more reactive OAT than when the enzyme is surrounded by free pyridoxal phosphate. OAT rapidly loses its enzymatic activity if maintained under mercaptan-free conditions. The importance of stabilizing the cysteinyl residues is revealed by the dramatic loss in activity upon treatment with N-ethylmaleimide. The sensitivity of M. sexta OAT to this reagent undoubtedly results from the fact that all of the cysteines are exposed at the surface and are able to react readily with N-ethylmaleimide.

Table IV

| Sample               | Enzymatic activity |
|----------------------|--------------------|
| Prior to dialysis    | 0                  |
| 72 h after dialysis  | 97                 |

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