Iso-orotate Decarboxylase:

Measurement of Kinetic Constants for Substrates and Inhibitors,

and Attempted Gene Isolation

by

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Abstract

Iso-orotate decarboxylase (IDCase) activity has been measured in protein lysates and ammonium sulfate fractionated suspensions prepared from wild type and mutant strains of *Neurospora crassa* using $^{14}CO_2$-displacement assays and UV-vis spectrophotometric assays. The activity of IDCase toward an alternate substrate, 2-thio-iso-orotate, which has an absorbance maximum outside the range of that for total protein, has been observed. The Michaelis-Menten constant for the original substrate, IOA, and alternate substrate, TIOA, have been measured to be K_m ~35 μM +/- 9 and K_m ~224 μM +/- 53 respectively.

5-nitrouracil is a strong inhibitor of IDCase activity, with a K_i too low for measurement by enzyme inhibition studies. Inhibtion of 5NU has been theoretically estimated to be ~0.5 nM. 5-nitrocytosine is a much less potent inhibitor, K_i ~26 μM. The K_i of its tautomeric form was also theoretically estimated to be ~2.5 nM giving rise to the possibility that the tautomer is responsible for inhibition activity towards IDCase. The inhibition and alternate substrate data is interpreted in a model for IDCase catalysis, in which a nucleophilic enzyme group makes a covalent attachment to the substrate prior to decarboxylation.

Purification using dye ligands as affinity chromatography resisns have yielded a definite negative result. IDCase did not preferentially bind to any column resisn tested.

Gene isolation experiments using bacterial complementation techniques have yielded a negative result.

Acknowledgements

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List of Symbols and Abbreviations

α	alpha
β	beta
ε	epsilon
°	degree Celcius
A	absorbance
g	gram
L	liter
M	molarity, moles per liter
mM	millimolar
ml	milliliter
nm	nanometer
μg	microgram
μl	microliter
μM	micromolar

Chapter 1 Introduction

Virtually all organisms synthesize pyrimidine nucleotides using the six step *de novo* pathway. The enzyme carbamyl phosphate synthetase catalyzes the condensation of 2 ATP, H_2O, HCO_3^- and the amino acid glutamine (as a nitrogen donor) to yield carbamyl phosphate. In the next four steps carbamyl phosphate is converted to orotidine-5′- monophosphate (OMP). OMP is further decarboxylated by the enzyme orotidine monophosphate decarboxylase (ODCase) to form uridine-5′-monophosphate (UMP). UMP can be directly incorporated into RNA or used as a general precursor for other pyrimidine nucleotides. These pathways include: conversion to cytosine nucleotides (CMP), which are incorporated into DNA or RNA; and conversion to thymidine monophosphate (TMP), which is incorporated into DNA (Figure 1a).

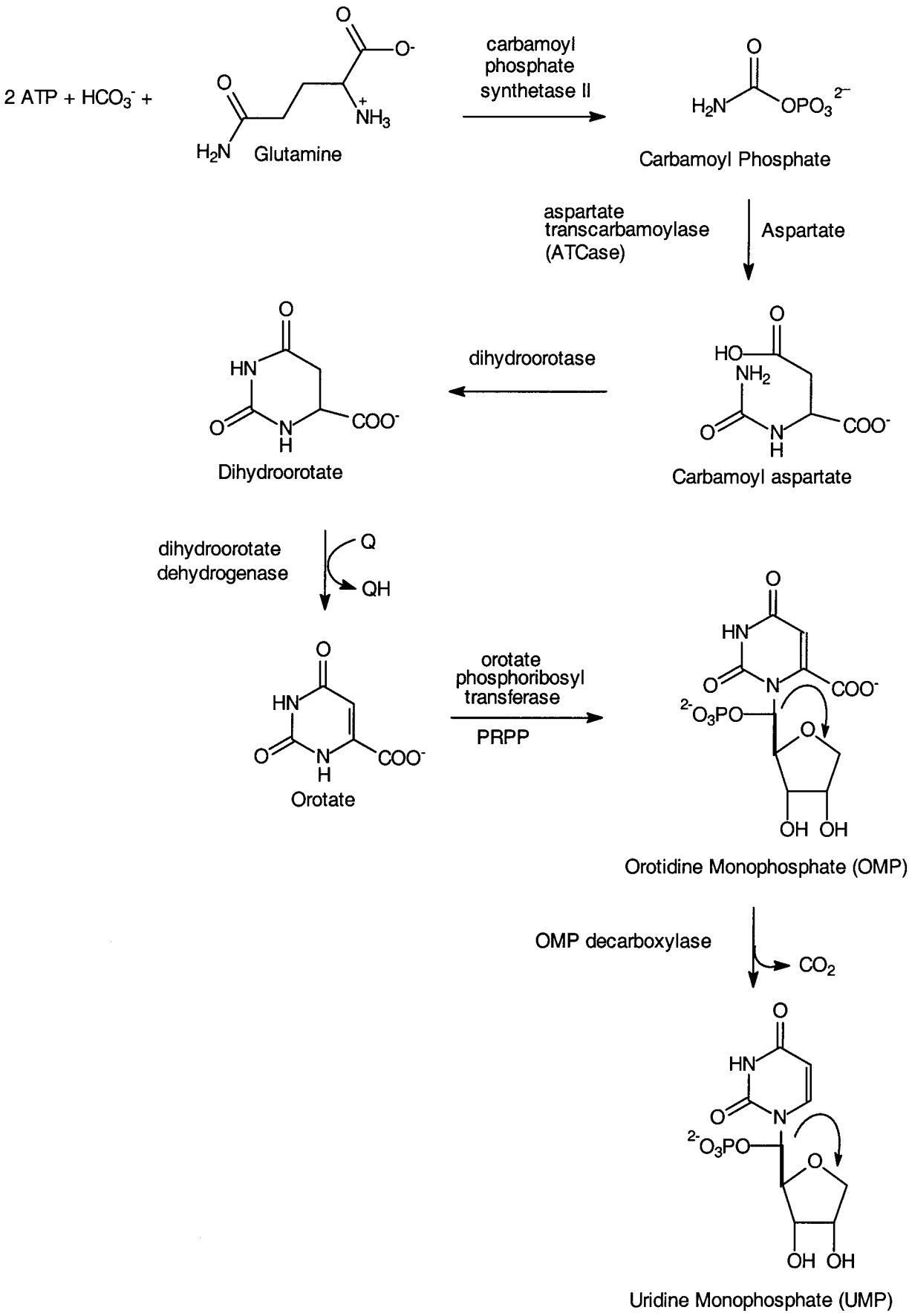
Thymidine nucleotide production is unidirectional. However, the pool of thymine-containing component is not. Studies indicate that some organisms are able to reuse thymidine utilizing a pyrimidine salvage pathway (Figure 1b,c). In this pathway, thymidine can be converted to uracil and eventually back to UMP for reentry in the total pyrimidine pool. This may lessen the need for pyrimidine biosynthesis through the *de novo* pathway, therefore conserving energy (ATP) and making the organism more metabolically versatile.

Thymidine is first oxidized to thymine ribonucleoside and then to thymine and ribose by the hydrolytic cleavage of the glycosidic bond. The remaining reactions involve the oxidation of thymine to uracil-5-carboxylate (referred to as iso-orotate, or IOA) catalyzed by thymine hydroxylase. Intermediate substrates formed are 5-

hydroxymethyl uracil and 5-formyluracil, respectively, the enzyme uses cofactors O_2, α-ketoglutarate, Fe^{++}, and the reducing agent ascorbate yielding oxidized succinate and CO_2 in a ratio of 1:1:1. [1]

The final step of this pathway and focus of this research involves the decarboxylation of iso-orotate to uracil catalyzed by uracil-5-carboxylic acid decarboxylase, included in Figure 2 (referred to as iso-orotate decaboxylase, IDCase). Only limited investigation of IDCase was conducted in the early 1970's by Palmatier, McCroskey, and Abbott [2]. Studies show that IDCase catalyzes this decarboxylation reaction without cofactors and with high substrate specificity. In these experiments, IDCase was detected using ^{14}C labeled iso-orotate in the C2 or C7 position of the heterocycle and also on the carboxylate separately which yielded ^{14}C-uracil and $^{14}CO_2$ respectively.

Figure 1a: *de novo* Pyrimidine Biosynthetic Pathway[1]


2 ATP + HCO3- +
Glutamine
carbamoyl phosphate synthetase II
Carbamoyl Phosphate
aspartate transcarbamoylase (ATCase)
Aspartate
Carbamoyl aspartate
dihydroorotase
Dihydroorotate
dihydroorotate dehydrogenase
Q
QH
Orotate
orotate phosphoribosyl transferase
PRPP
Orotidine Monophosphate (OMP)
OMP decarboxylase
CO2
Uridine Monophosphate (UMP)

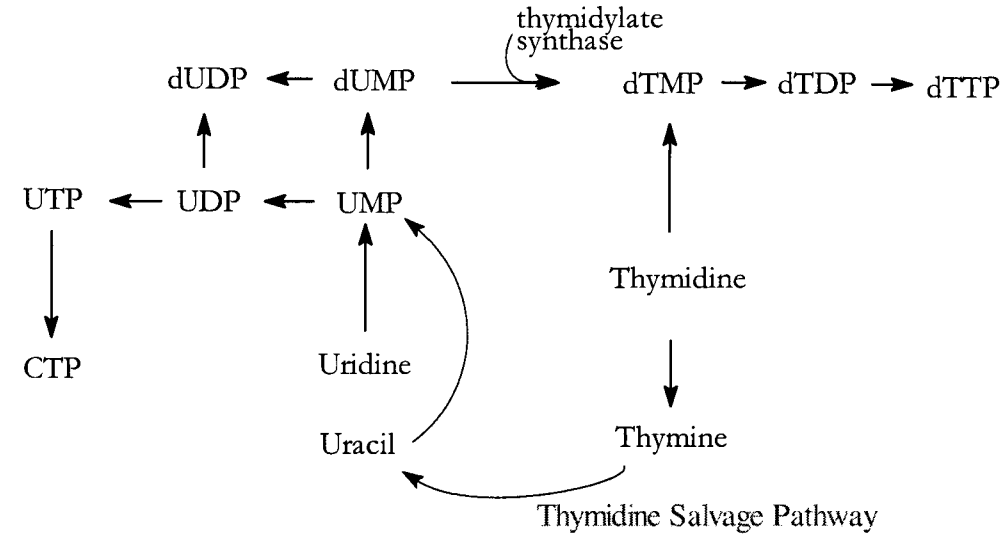


Figure 1b: Thymine Salvage pathway in *N. crassa* allows thymine to enter the total pyrimidine pool.

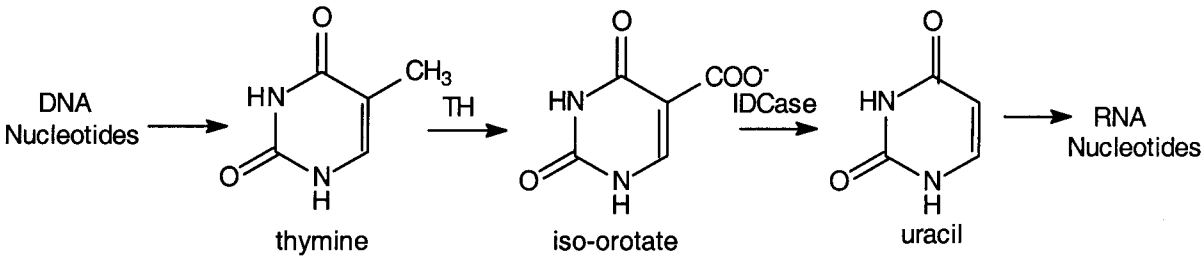


Figure 1c: Conversion of thymine to uracil by thymine hydroxylase (TH) and iso-orotate decarboxylase (IDCase) via the thymine salvage pathway in fungi.

Thymidylate Synthase: Proposed Mechanistic Model for IDCase

Enzymes involving nucleophilic attachment at C6 of pyrimidines have precedent in the literature. The mechanism of thymidylate synthase (Figure 2) has been well described and used as a model for the decarboxylation mechanism of iso-orotate to uracil by IDCase (Figure 3). In the irreversible synthesis of dTMP, dUMP is methylated by TS using N^5,N^{10} – methylenetetrahydrofolate (THF) as the methyl donor which is reduced to dihydrofolate. This mechanism can be explained in four events[1]

1. Activation of the C5 position of dUMP

A thiolate group of Cys 198 of TS undergoes the nucleophilic attack at C6 causing the C5 position to become more electron rich.

2. Formation of TS-dUMP-THF complex

The C5 position of dUMP attacks the N5 methylene of THF yielding a ternary covalent complex.

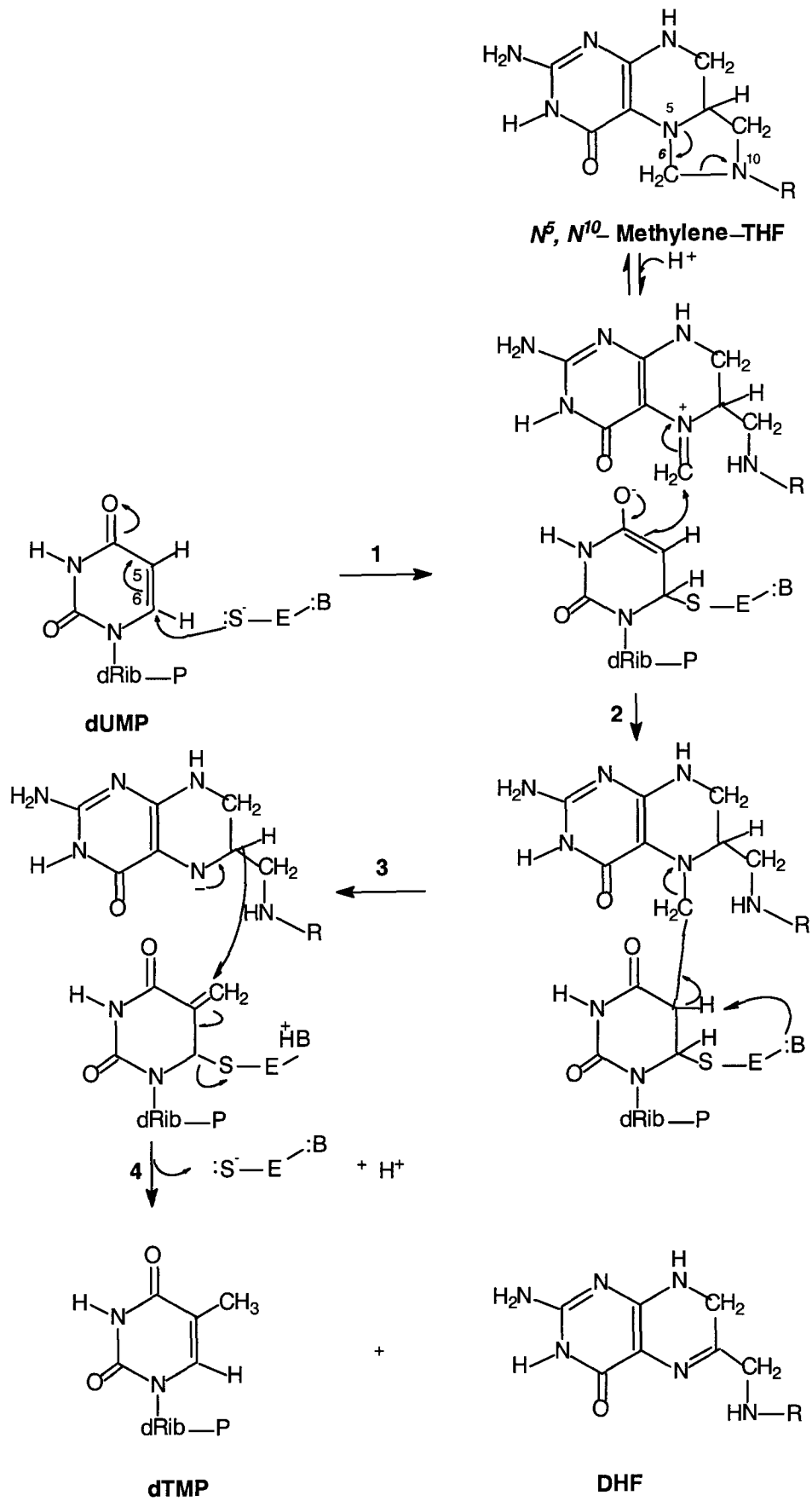
3. Elimination of THF Cofactor

In an internal nucleophilic attack, an enzyme base at C5 abstracts an acidic proton at C6. This disrupts the TS-dUMP-THF complex thus freeing THF. The abstracted proton is exchanged with the solvent.

3. Formation of dTMP

Dihydrofolate is formed when a H^+ adjacent to N5 of THF is lost via a H^- transfer. The hydride ion converts the methyene to a methyl group resulting in enzyme release.

Figure 2a: Catalytic Mechanism of Thymidylate Synthase (TS) [1]


N^5, N^{10}– Methylene–THF
dUMP
dTMP
DHF
1
2
3
4
dRib—P

Evidence supporting this mechanism was found in mechanism-based inhibition studies of TS. These inhibitors resemble the substrate and undergo partial catalysis of the complex formation step, thus inactivating the enzyme. By understanding how TS binds to these inhibitors, specifically the nucleophilic attack at C6, investigators were able to deduce the binding interaction of TS and dUMP.

An example of such an inhibitor is 5-fluoro-2′-deoxyuradine-5′-monophosphate (FdUMP). In event 3 (Figure 2) an internal nucleophilic attack occurs between an enzyme base and C5. The enzyme base of TS-FdUMP-THF complex cannot abstract the fluorine atom as F^+ to free itself from the complex thus inactivating TS.

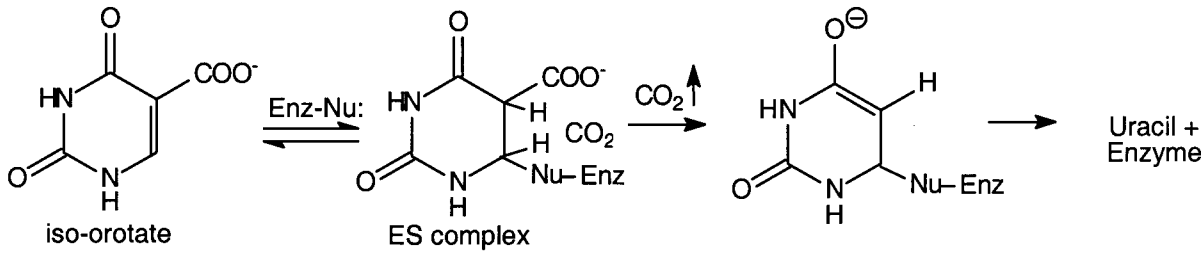


Figure 2b: Proposed mechanism of action for IDCase: Nucleophilic attack at C6 promotes decarboxylation of α carbon.

Inhibition of Enzyme Activity

A substance that decreases the velocity of an enzyme-catalyzed reaction can be categorized as an inhibitor. Similar to the mechanistic studies of thymidylate synthase, inhibition studies can be employed to determine kinetic parameters as well as mechanistic information about enzymes. If a compound is a strong inhibitor, its chemical properties can be related to a proposed mechanism for the enzyme it inhibits. In the enzymological investigation of IDCase two compounds were examined, 5-nitrouracil (5NU) and 5-nitrocytosine (5NC).

5-nitrouracil has been previously described in the literature as a strong competitive inhibitor of IDCase [3]. 5-nitrouracil and iso-orotate are related compounds with different groups at position C5 of the heterocycle. C5 of iso-orotate is occupied by a carboxylate where there exists an electron withdrawing nitro-group on the 5NU. The electrophilic nature of the C6 position of both molecules allows for the attack of an enzyme nucleophile. In comparison with the TS mechanism, the enzyme cannot free itself from the inhibitor-enzyme complex because the NO_2 moiety on 5NU is an unfavorable leaving group whereas CO_2 is easily removed. Measurement of kinetic parameters of 5-nitrouracil, specifically the inhibition constant K_i, was attempted.

The second inhibitor used in these experiments was 5-nitrocytosine. As with 5-nitrouracil, this potential inhibitor has an electrophilic C6 suitable for nucleophilic attack by IDCase. It is also similar in size to iso-orotate allowing for active site entrance.

Affinity Chromatography

A characteristic similarity of several proteins is their ability to tightly bind specific molecules in a reversible fashion [1]. This property can be used to purify such proteins using affinity chromatography. A ligand containing many functional groups that is presumed to bind to the protein of interest is covalently attached to an inert, nonporous stationary matrix. A protein solution is then passed through the chromatographic matrix, and the enzyme of interest binds to the ligand while other components of this solution are washed through. The protein can then be recovered by changing elution conditions such as increasing salt concentration or altering pH. This causes the protein-ligand complex to become unstable allowing for protein elution.

IDCase purification was attempted using chromatographic techniques used to purify orotidine decarboxylase (ODCase) by Bell and Jones [4]. In this procedure, an ammonium sulfate precipitated protein lysate solution was column fractionated using Affi-Gel Blue affinity chromatography resin. A 12.3 fold increase in specific activity was observed for the chromatographic method. Approximately 40% of ODCase activity was recovered from the column. In this study, several commercially available dye matrix columns were used in the attempt to find a suitable matrix for purification.

$^{14}CO_2$ Radioassay

Quantification of IDCase activity was determined using ^{14}C-labeled iso-orotate. Assay protocol was based on the original technique in detecting the conversion of iso-orotate to uracil and CO_2 (Palmatier *et al*) [2]. A reaction mixture containing [7-^{14}C]IOA and ammonium sulfate fractionated protein lysate were incubated at 30°C for various time periods. The reaction was quenched upon hypodermic needle injection of 2 M

hydrochloric acid. Liberated $^{14}CO_2$ was collected on base- soaked filter paper which was positioned in a reservoir suspended from a rubber septum. After a one hour incubation period, the filter paper was removed and dried in a vacuum oven for approximately 45 minutes then counted in scintillation fluid medium. The counts per min (cpm) detected were directly correlated to the amount of product formed and used to calculate enzyme activity (Table 1). Due to the sensitivity of the radioassay, this technique was very useful in determining activity in even diluted protein lysates since a 5% conversion could easily be detected. In addition to enzyme activity determinations, the radioassay was used in determining kinetic and inhibition parameters of IDCase.

Spectrophotometric Assay

Ultraviolet spectroscopy was used in determining alternate substrate activity of 2-thio-iso-orotate (TIOA). Since TIOA has an absorbance maximum outside that of total protein, it lends itself to a direct spectrophotometric assay of decarboxylation activity. Assay development was similar to that used in ODCase studies by the Lieberman group [5].

Reaction mixtures containing various concentrations of TIOA and protein lysate solution were assayed at 334 nm. The observed decrease in absorbance from each assay was measured over 10 second intervals and used to calculate kinetic parameters (TABLE 2).

Bradford Assay

Enzyme concentration in μg/μl was spectrophotometrically determined using bovine serum albumin as the standard protein curve. Results were used to calculate specific activity of IDCase in radioassays and UV spectroscopy assays.

Table 1: ^{14}C-Radioassay Calculation Examples

Term	Definition of Term	Mathematical Operation	Example[a]
cpm	Amount of $^{14}CO_2$ detected per minute	cpm/min	193/10 = 19.3
min	Time point length of each assay		
cpm/nmol	Conversion factor[b] 200. Used to obtain nmol of activity in each assay	cpm / (cpm/nmol) = nmol	193/200 = 0.965
cpm/min/μL	The rate of conversion to product for each specific assay volume	(cpm/min) / μl	19.3/25 = 0.772
nmol/min/mg	The rate of conversion of substrate to product dependent upon concentration of enzyme in reaction mix[c], specific activity of the enzyme	(nmol/min) / mg	0.0965/0.068 = 1.41

[a]Calculations from wild type strain 74A, 50μM [7-^{14}C]IOA, 1.25mM Tris pH 7.4
[b]Specific activity of $^{14}CO_2$
[c]IDCase concentration from $(NH_4)_2SO_4$ lysates measured using Bradford Assay

Table 2: UV-vis Spectroscopy Calculations for Determination of Enzymatic Reaction Rate

2-thio-iso-orotate[a] Concentration μM	ΔA/min[b]	Mathematical Operations, calculation of rate note: 12,800 = $\Delta\varepsilon_{334}$
50	0.0093	0.0093 / 12,800M^{-1} x 0.001L[c] x10^9nmol/min = 0.625
100	0.0196	0.0196 / 12,800M^{-1} x 0.001L x 10^9nmol/min = 1.2109
150	0.0214	0.0214 / 12,800M^{-1} x 0.001L x 10^9nmol/min = 1.5781
200	0.0254	0.0254 / 12,800M^{-1} x 0.001L x 10^9nmol/min = 1.9844

[a]Calculations from assay using 500 μM TIOA in 25 mM Tris pH 8.0

[b] ΔA/min was determined from the slope of individual "A_{334} vs. Time" plots for corresponding concentrations of TIOA

[c]Total volume of each assay

This project will attempt to uncover more of the enzymological properties of IDCase. Two enzymatic assays are described, kinetic parameters such as K_m and K_i are measured, and a proposed mechanism is presented involving nucleophilic attack at C6. Enzyme inhibition data supports this mechanism. Attempts at protein purification, using ^{14}C assays to detect the presence of the enzyme, and experiments on genetic complementation screening will also be described.

Chapter 2 Preparation of IDCase from *Neurospora crassa*

Introduction

Neurospora crassa: IDCase Protein Source

IDCase enzyme was obtained from *N. crassa*, an organism included in the ascomyceteous fungi category. Two strains of this organism were used as a protein source: Wild type 74A, which possess as all the enzymes of the thymine salvage pathway and the *de novo* pathway for synthesizing pryimidines, and mutant strain KFSC #2203 [6], which lacks complete thymine and *de novo* pathways. Strain #2203 contains mutations in *pyr*-4 gene which encodes for ODCase, the enzyme responsible for catalyzing OMP to UMP in the last step of the *de novo* pathway; and the gene *uc*2, which encodes for thymine 2′ hydroxylase, the enzyme that catalyze the conversion of thymidine to ribothymidine and deoxyuridine to uridine. Since 2203 does not contain functional ODCase, and is therefore is unable to synthesize pyrimidines for RNA and DNA biosynthesis, uracil was added to minimal growth media as a supplemental pyrimidine source for this strain.

Growth of *N. crassa* wild type and mutant strains were done in the same manner. A loop of frozen stock was used to inoculate Horowitz media and agar mix (strain #2203 required URA) with growth appearing in 3-5 days. The conidia from one flask was suspended in a small volume of sterile media and used to inoculate a larger volume of this liquid media. After incubation for a designated period of time the tissue was harvested by vacuum filtration, weighed, and used immediately.

IDCase lysates were prepared using the same protocol for each strain. The tissue was transferred to a solution of ice-cold buffer and protease inhibitors and subjected to mechanical agitation to break the cell membranes. The suspension was treated

intermittently with ice baths to maintain a low temperature range. After centrifugation to remove debris, the lysates were subjected to ammonium sulfate fractionations to remove unwanted proteins and to concentrate the enzyme as much as possible. The lysate pellet was resuspended in buffer and dialyzed three times to remove excess salt and metabolites.

Materials and Methods

Preparation of Protease Inhibitors

Three protease inhibitors (Sigma) were used in the lysis buffer for *N. crassa* cultures phenylmethylsulfonylfluoride (PMSF), pepstatin A, and leupeptin. A 100 mM stock solution of PMSF was prepared by dissolving 0.0871g in 5 ml absolute ethanol (Fisher). A 1mM working solution was prepared by diluting 50 µl of 100 mM PMSF in 5 ml absolute ethanol. 1.46 mM of pepstatin A was prepared by dissolving 0.005g in 5 mL dIH_2O. 2.10 mM leupeptin was prepared by dissolving 0.0056g in dIH_2O. All solutions were stored at 4 °C until use.

N. crassa Growth and Culture

Strains 74A and 2203 were grown using the same protocol. 500 ml of Horowitz media was prepared (Table 3a) and autoclaved (Uamato SM 32) in 25 ml portions in 125-mL Erlenmyer flasks. 0.375g of bactoagar was added to each flask and covered with cotton and aluminum foil before autoclaving. Flasks were used immediately upon cooling or stored with foil intact at room temperature for a maximum of 3 weeks. 50 µl of 20mg/ml uracil was added to those flasks used to grow strain #2203. Using a flame-sterilized loop,

a small portion of frozen stock 74A or 2203 (KFSC) was spread across formed media and stored with cotton loosely covering the flasks at room temperature (~25 °C). Within 3-5 days orange, cotton like conidia had appeared. Only 1 flask was inoculated with frozen stock at a time. Subsequent inoculations were made using another sterile loop of conidia from the original. Conidia produced from these appeared in 2-3 days.

1X Westergaard's [7] media plus 2% sucrose was prepared from 10X Westergaard's (Table 3b) and used in the following. 20 ml of room temperature sterile media was added to one flask containing orange conidia with vigorous swirling for ~5 minutes. Equal amounts of inoculum were transferred with a sterile pipette to two 500 ml Erlenmeyer flasks containing 125 ml sterile media. For strain #2203 growth, 750 µl of 20mg/ml uracil was added to the 1X Westergaard's prior to sterilization of the media. Flasks were covered loosely with cotton and allowed to incubate with vigorous shaking at 30 °C for 18-24 hours (Fisher Isotemp 200 series model 255D). Specific activity of IDCase was variable between this range of time periods. Incubation period was decided based on amount of tissue appearing at each hour.

Harvest and Preparation of Tissue

Orange, rubbery tissue appearing within 18-24 hours of inoculation was harvested by manual vacuum filtration using a side arm flask, Buchner funnel, filter vac, and Whatman filter disks. Once residual media was removed, the tissue was weighed and frozen with liquid nitrogen or dry ice and stored at -70°C (VWR Scientific freezer). In most experiments, however, tissue was used immediately upon harvesting. The amount of tissue resulting from each harvest varied from 2.5-4.5g. In a sterile 50 ml Fisher brand conical centrifuge tube, 5g of tissue was suspended 1:3 ice in cold GDH buffer (10 mM EDTA, 33 mM Tris, and 0.007% β-mercaptoethanol, 13.3 µl 1.46 mM pepstatin A, 6.8 µl

2.10 mM leupeptin, and 40 µl 1 mM PMSF. The cell membranes were disrupted at 30 second intervals using a tissue homogenator (Biospec model 985-370) at the maximum setting of 30,000 rpm. The tissue was cooled on ice for 30 seconds between each agitation. Cell suspension was transferred to Beckman polystyrene tubes and centrifuged at 25,000 rpm (Beckman L8-55 Ultracentrifuge) for 20 minutes and 4 °C. The resulting cell pellet was discarded and the supernatant was transferred to a 50 ml beaker for $(NH_4)_2SO_4$ treatment.

Table 3a: Horowitz Media

Compound (Sigma)	Amount
Potassium tartrate	5.0 g
$NaNO_3$	4.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 2H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
glycerol	0.05 g
hydrolyzed casein	0.25 g
yeast extract	5.0 g
malt extract	5.0 g
dissolved in 950 mL dIH_2O and	
diluted to 1L	

Table 3b: Westergaard Media for Tissue Culture[a]

Compound (Sigma)	Amount
KNO_3	5.0 g
NH_2PO_4	5.0 g
$MgSO_4 \cdot 7H_2O$	2.5 g
NaCl	0.5 g
$CaCl_2 \cdot 2H_2O$	0.5 g
Trace Elements[b]	0.5 ml
Biotin (50X)	0.5 ml
diluted to 500 ml with dIH_2O	
stored at 10°C	

[a]1X prepared with addition of 1g sucrose/50ml media
[b]See Table 3b for preparation

Table 3c: Trace Element Solution

Compound (Sigma)	Amount
Citric acid	5.0 g
$ZnSO_4 \cdot 7H_2O$	5.0 g
$Fe(NH_4)_2(SO_4) \cdot 6H_2O$	1.0 g
$CuSO_4 \cdot 5H_2O$	0.25 g
$MnSO_4 \cdot H_2O$	0.25 g
H_3BO_3 anhydrous	0.05 g
$Na_2MoO_4 \cdot 2H_2O$	0.05 g
$CDCl_3$	1.0 ml
dissolved in 80 ml dIH_2O and diluted to 100 ml	

Preparation of IDCase Lysates by Ammonium Sulfate Precipitation

Purification grade $(NH_4)_2SO_4$ (Amresco) was added (see to Table 4 for gram to volume addition) in 5 minute increments for ~20 minutes with constant stirring at 10 °C. The mixture was allowed to homogenize for ~1 hour with continued stirring at 10 °C, after which time it was centrifuged at 25,000 rpm for 20 minutes at 4 °C. The resulting supernatant was subjected to $(NH_4)_2SO_4$ addition until 90% saturation was achieved. After ~ 1 hour stirring at 4 °C the mixture was centrifuged at 25,000 rpm for 20 minutes at 4 °C. The resulting pellet was resuspended in 750 µl of buffer I (50 mM Tris pH 8.0, 2 mM EDTA, and 10% glycerol, Fisher) and transferred to a dialysis bag (Fisher spectrpor molecular porous membrane). The protein was dialyzed 1:1000 in Tris-glycerol buffer (50mM Tris pH 7.8, 2mM EDTA, 10% gycerol) 1.5-3 hours and ~12 hours in fresh Tris-glycerol at the same ratio. A third buffer change occurred at the same ratio for an additional 1.5 hours. The protein lysate was transferred to a sterile Eppendorf tube and spun in an Eppendorf centrifuge 5415C to remove any residual salt or debris. The lysate was kept on ice and immediately assayed.

Final Concentration of Ammonium Sulfate - % saturation

	20	45	60	90
Initial % [$(NH_4)_2SO_4$]	Solid $(NH_4)_2SO_4$ added to 100 ml of solution			
0	10.6	25.8	**36.1**	60.3
15	2.6	17.2	27.1	50.3
30		8.6	18.1	40.2
45		0	9.0	30.2
60			0	**20.1**

Table 4: Ammonium Sulfate Fractionation, 60% and 90%[8]

Quantitation of IDCase Lystates

The protein concentration of ammonium sulfate fractionated lysates were determined using the Bradford assay. Solutions of 5, 10, 15, 20 µl of 0.5mg/ml bovine serum albumin (stock 2mg/ml in 0.9% NaCl with sodium azide, Pierce) were added to separate nonsterile Eppendorf tubes and the volume brought to 100 µl with 0.15 M NaCl. A blank containing 100 µl 1 0.15 M NaCl was also prepared. 2-5 µl of protein lysate was transferred to a nonsterile Eppendorf tube and the volume brought to 100 µl with 0.15 M NaCl. 1 ml of Coomassie Blue solution (Pierce) was added to each tube and subjected to vortex for ~30 seconds. The solutions were then allowed to stand for a minimum of 2 minutes and maximum of 10 minutes. The A_{596} was measured on a Hewlett Packard 8452A diode array spectrophotometer and the BSA samples were used to construct a standard curve. The total protein of unknown samples in µg were determined from the graph and used to calculate protein concentration (µg/µl) and in turn specific activity for each radioassay or spectrophotometric assay performed at a later time. Refer to Figure 3 for a representative sample graph and calculation of protein concentration.

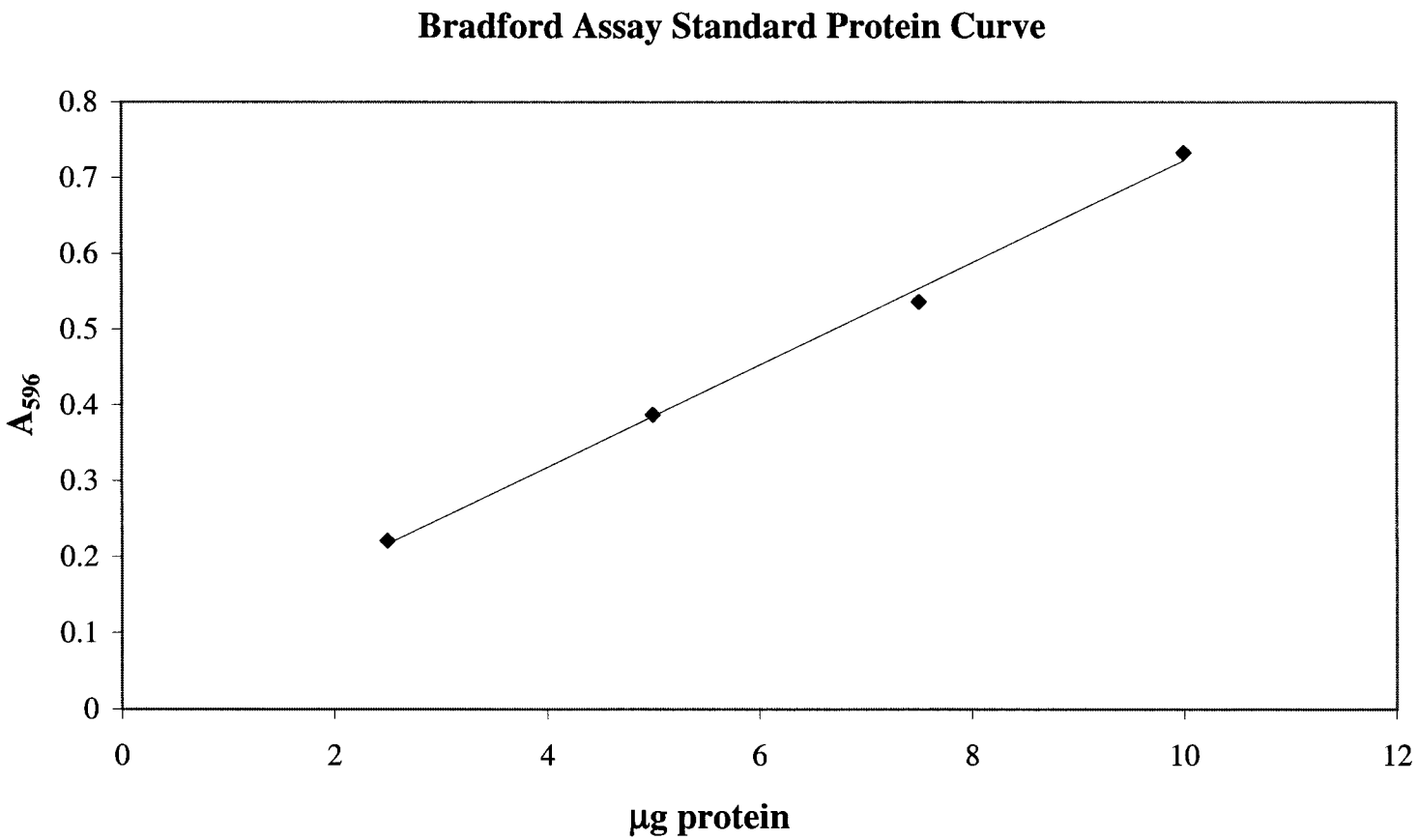


Figure 3: Standard protein curve using 0.5 mg/ml BSA, IDCase concentration (in µg) measured using equation of the line

Chapter 3 Kinetic Parameters of Iso-orotate Decarboxylase

Introduction

Determination of Kinetic Constants of Iso-orotate Decarboxylase for Iso-orotate and 2-thio-iso-orotate

Kinetic parameters of the original substrate of IDCase, iso-orotate, and an alternate substrate, 2-thio-isoorotate, have been determined. These kinetic constants were determined for the enzyme in an impure preparation. Assay development included two techniques such as $^{14}CO_2$-displacement and UV-vis spectrophotometric experiments.

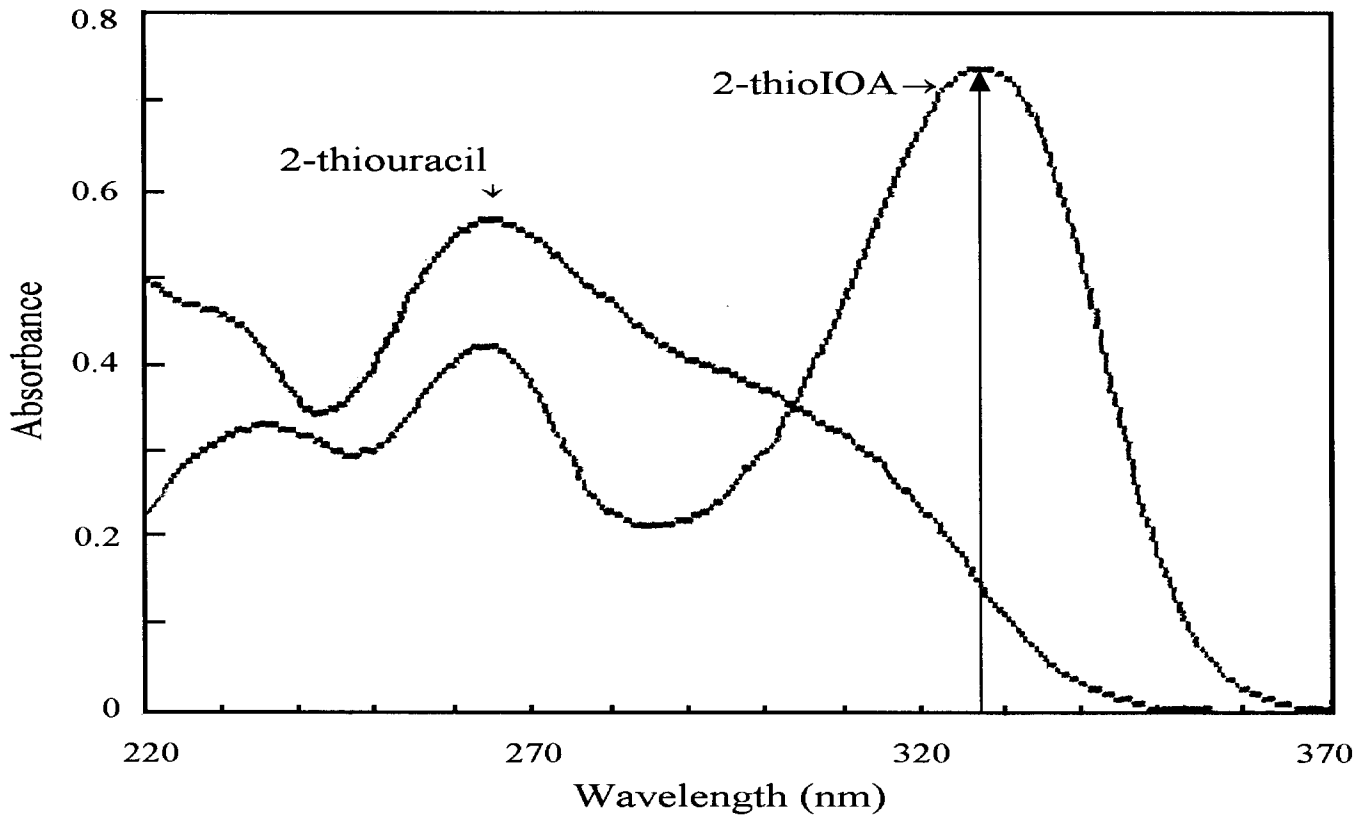
The Michaelis-Menten kinetic constant, K_m, is the concentration at which the enzyme operates at half-mixed velocity. The K_m was determined for IOA using $^{14}CO_2$-displacement assays. In these experiments, the volume of protein lysate and final assay volume remained constant while substrate concentration varied. Each reaction vial contained radiolabeled IOA, buffer, and diluted to a constant volume with dIH_2O. Each vial was sealed with a rubber septum with a plastic well extending from the bottom that contained base soaked filter paper. At the reaction start time, IDCase was added to each vial which was then placed in a shaking incubator for a predetermined period of time. Each reaction was quenched with acid with continued incubation. This was to ensure all $^{14}CO_2$ was absorbed onto the base soaked wick. All wicks were dried to remove base solution and counted to determine amount of radioactivity produced from each reaction. The amount of $^{14}CO_2$ detected after varied reaction times was directly related to the amount of product formed (1:1 ratio). Counts of radioactivity per minute (cpm) were used to calculate the velocity of each reaction (see Table 1) and plotted against IOA concentration in a double-reciprocal plot. This graphical representation is referred to as a Lineweaver Burk kinetic plot. The value of K_m, the substrate concentration at which

velocity is half of the theoretical maximum, can easily be determined from the linear graph.

The K_m for 2-thio-iso-orotate was measured using UV-vis spectroscopy. The conversion of TIOA to TURA is based on the disappearance of absorbance, from 334 nm to 280 nm for TIOA, which allows for observable enzymatic catalysis (Figure 4) over a period of time. Similar to the radioactive assays, the amount of protein lysate and total assay volume remained constant while varying the substrate concentration. Buffer and protein lysate solution were diluted up to a constant volume with dIH_2O and used to blank the spectrophotometer. Upon addition of TIOA, absorbance readings were taken for 10-second intervals at 334 nm. This procedure was repeated with 3 increasing substrate concentrations. Decreasing absorbance values were used to calculate the rate of reaction in nmol/min (see Table 2) and plotted against substrate concentration in a double reciprocal Lineweaver Burk Plot. The K_m was determined from the –x intercept of the graph, representing $-1/K_m$.

The kinetic inhibition constants, K_i, for 5-nitrocytosine and 5-nitrouracil were also measured using $^{14}CO_2$-displacement assays. In these experiments, the amounts of ^{14}C-IOA, buffer, and protein lysate remained constant while varying inhibitor concentrations. The general experimental procedure for using $^{14}CO_2$-displacement assays was described previously in this text.

Spectral Difference Between 2-thioIOA and 2-thiouracil Allows Enzyme Assay at 334 nm



Spectral difference is greatest at l = 334 nm; $\varepsilon_{334} = 12400\ M^{-1}$

Figure 4: Graphical Representation of 2-thiouracil and 2-thioIOA spectra

Materials and Methods

Preparation of Substrate and Inhibitor Solutions for Kinetic Experiments

^{14}C-IOA [^{14}C] formaldehyde was purchased from Dupont-NEN. [7-^{14}C] iso-orotate was synthesized in a two step reaction described by Cline and Fink [8]. Uracil and [^{14}C] formaldehyde were reacted under basic conditions yielding [7-^{14}C] hydroxymethyluracil. Oxidation of HMU by PtO_2 addition yielded [7-^{14}C] iso-orotate used for all radioactive assays. To ensure radioactive counts detected were the result of ^{14}C-IOA conversion to uracil and $^{14}CO_2$, excess CO_2 was removed by addition of microliter amounts of 2 M HCl.

2-thio-iso-orotate All reagents were purchased from Fisher Chemical Company with the exception of 5-carboxy-2-thiouracil (TIOA) which was obtained from Sigma. A 500 µM solution in 25 mM Tris pH 7.4 was prepared by dissolving 0.002152g of TIOA in 25ml of dIH_2O and 625 µl of 1.0 M Tris buffer. The solubility of TIOA in water was increased by the addition of 3 drops of 2 M NaOH. The exact concentration was determined using UV-vis spectroscopic methods. The absorbance of a 1:10 solution of TIOA containing 850 µl dIH_2O, 50 µl Tris pH 7.4, and 100 µl of ~500 µM TIOA solution was measured over the range of 220nm-370nm. Hewlett Packard model 8452A diode array spectrophotometer was used in the quantification. The concentration was calculated from Beer's law where $A=\varepsilon$ x b x c. A_{328} is the absorbance at maximum wavelength 328nm, ε is the extinction coefficient or molar absorptivity for TIOA determined to be 15,500 L/mol·cm [10], b is the path length of the quartz cell (= 1cm) and c is the concentration expressed in mol/L. At maximum wavelength, $A_{328} = 0.8543$. Using Beer's law equation,

$$A_{328} = \varepsilon \times b \times c$$

$$0.8543 = 15{,}500\ \text{L/mol·cm} \times 1\ \text{cm} \times c$$

$$c = 5.51161\text{E-}05\ \text{M}$$

Converting this result to μM and multiplying by a factor of 10 (1:10 dilution), the final concentration of TIOA solution was determined to be 551 μM.

5-nitrocytosine 5NC was obtained form D.Yun, Department of Chemistry Youngstown State University. Concentrations in the range of 20 μM to 0.02 μM were prepared by dilution. A more concentrated solution of 5NC was prepared by dissolving 0.0079g of compound in 25 ml of diH_2O. This solution was also diluted 1:10 with 850 μl diH_2O, 50 μl Tris pH 7.4, and 100 μl 5NC. The concentration was determined using the same spectrophotometric method for TIOA and the Beer's law equation where A_{354} = 1.263 and ε = 16,218 [11]. This concentration was calculated to 1.40 mM. Refer to Table 5 for preparation of 200 μM and 20 μM 5NC stock solutions.

5-nitrouracil Solutions of 5NU (Sigma) were prepared using the same method as 5NC where maximum absorbance was recorded at A_{342}. Refer to Table 5 for preparation.

Table 5: Inhibitor Solution Preparation for Kinetic Assays

Compound	Preparation	Estimated Conc.	λ_{max} nm	$A_{\lambda max}$	Calculated Conc.
5-NC	1.433 ml of 1.40 M diluted to 10 ml with dIH_2O	200 μM	354	0.3482	215 μM ~200 μM
5-NC	932μl of 215 μM diluted to 10 ml with dIH_2O	20 μM	354	0.0302	18.6 μM ~20 μM
5-NU	0.006283g 5NUra dissolved in 20 ml dIH_2O	2 mM	342	2.417	1.51 mM
5-NU	1.33 ml of 1.51 mM diluted to 11 ml	200 μM	342	0.3472	217 μM ~200 μM
5-NU	922 μl 217 μM diluted to 10 ml	20 μM	342	0.0385	24 μM ~20μM

Measurement of the Michaelis-Menten Constant: $^{14}CO_2$-Displacement Assays (IOA)

500 μM frozen stock (5000cpm/50μl) ^{14}C-IOA was thawed and subjected to vortex to thoroughly redissolve precipitated material. In each of the 24 assay vials used, 25 μL Tris pH 7.4 was added along with sufficient volume of diH_2O to bring the final volume to 500 μl. Vials numbered 1-4 contained 10 μl $^{14}CO_2$-IOA for 10 μM, vials numbered 5-8 contained 20 μl $^{14}CO_2$-IOA for 20 μM, vials numbered 9-12 contained 30 μl $^{14}CO_2$-IOA for 30 μM, vials numbered 13-16 contained 50 μl $^{14}CO_2$-IOA for 50 μM, vials numbered 17-20 contained 80 μl $^{14}CO_2$-IOA for 80 μM, and numbered 21-24 contained 100 μl $^{14}CO_2$-IOA for 100 μM. The reactions were initiated by the addition of 25 μl of IDCase ammonium fractionated lysate (procedure explained previously). For each substrate concentration, reactions were allowed to incubate with moderate shaking at 30 °C for duplicate time points of 10 and 20 minutes (Precision Scientific metabolic shaking incubator). After this time the reactions were quenched with hypodermic addition of 200 μl of 2 M HCl. Each vial was allowed to further incubate for 1.5 hours. The wicks were removed with ethanol/dIH2O rinsed tweezers, placed into a sectioned drying plate, and dried at 80 °C for ~45 minutes. Each wick was then placed in ~6 mL of scintillation medium (ScintiSafe™ Econo 1) and counted for 5 minutes (Packard Tri-Carb 1900CA Liquid Scintillation Analyzer). The vials containing scintillation fluid only were counted as blanks prior to use.

Measurement of Michaelis-Menten Constant: UV-vis Spectroscopy (TIOA)

Fractionated protein lysate (method previously described), Tris solution and diH_2O were mixed and used to blank the instrument. To initiate the reaction, 50 μl of 500 μM TIOA in 25 mM Tris pH 7.4 was added (total assay volume 1 ml) and A_{334} was recorded for 2

minutes at 10-second intervals. Three additional assays were performed by varying TIOA concentrations: 100, 150, 200 µl while retaining final assay volume as 1 ml. The rate of each reaction was determined (see Table 2) and used to construct Lineweaver Burk plots to determine the value of K_m.

Inhibition Measurement of 5-nitrocytosine and 5-nitrouracil: $^{14}CO_2$-Displacement Assays (IOA)

Initial Estimation of K_i (5-nitrocytosine) A 500 µM frozen stock (5000cpm/50µl) of ^{14}C-IOA was thawed and subjected to vortex just before use. An initial experiment was performed to test if 5NCyt had inhibitory properties. Radioassays were performed using the same procedure as described for K_m determination of IOA. 8 assay vials contained 50 µl ^{14}C-IOA, 25 µl Tris pH 7.4, and dIH_2O to bring the final assay volume to 500 µl. 200 and 20 µM stock 5NC stock solutions were added to give [I] = 20, 2, 0.2, 0.02 µM for duplicate vials. The reactions were initiated by the addition of ammonium sulfate fractionated protein lysate (method previously described). Each reaction was allowed to incubate at 30 °C for 1 hour after quenching. Refer to Table 6a for assay conditions.

Determination of K_i using Multiple Assay Measurements (5-nitrocytosine) Thirty-two separate assay vials were used in this experiment containing constant volumes of ammonium sulfate fractionated protein lysate (method previously described). Volumes of 200 µM 5NCyt and ^{14}C-IOA were added so that for each substrate concentration, where [S] = 15, 30, 60, 100 µM, inhibitor concentrations were varied in 10 µM increments, where [I] = 0, 10, 20, 30 µM. Each reaction was allowed incubation for

duplicate 10-minute time points before quenching and continued incubated for 1 hour. Refer to Table 7 for assay conditions.

5-nitrouracil Initial Experiment A 500 μM frozen stock (5000cpm/50μl) of ^{14}C-IOA was thawed and subjected to vortex just before use. The K_i determination of 5NU was acheived using the same methods described for K_i determination of 5NC (experiment 1). 200 and 20 μM stock solutions of 5NU were used to obtain final assay concentrations of [I] = 20, 2, 0.2, 0.02 μM. Refer to table 6b for assay conditions. Radioactive counts were not detected from these assays.

Table 6a: Initial Kinetic Inhibition Assay (5NC)

Compound	Volume µl			
	[I] =20 µM	**[I] =2 µM**	**[I]=0.2 µM**	**[I] =0.02 µM**
^{14}C-IOA	50	50	50	50
Tris pH 7.4	25	25	25	25
IDCase lysate	25	25	25	25
dIH_2O	350	395	350	395
5-nitrocytosine	50 $_{200\ \mu M\ stock}$	5 $_{200\ \mu M\ stock}$	50 $_{20\ \mu M\ stock}$	5 $_{20\ \mu M\ stock}$

Table 6b: Initial Kinetic Inhibition Assay (5NU)

Compound	Volume µl			
	[I] =20 µM	**[I] =2 µM**	**[I]=0.2 µM**	**[I] =0.02 µM**
^{14}C-IOA	50	50	50	50
Tris pH 7.4	25	25	25	25
IDCase lysate	25	25	25	25
dIH_2O	350	395	350	395
5-nitrouracil	50 $_{200\ \mu M\ stock}$	5 $_{200\ \mu M\ stock}$	50 $_{20\ \mu M\ stock}$	5 $_{20\ \mu M\ stock}$

Table 7: 5-Nitrocytosine[a] Kinetic Inhibition Assay
Multiple Assay Measurements

[S][b]=15μM

	I=0μM	I=10μM	I=20μM	I=30μM
Protein	50	50	50	50
Tris pH 7.4	25	25	25	25
^{14}CIOA	15	15	15	15
dIH_2O	410	485	460	435
5-NO_2-Cyt	0	25	50	75

[S]=30μM

	I=0μM	I=10μM	I=20μM	I=30μM
Protein	50	50	50	50
Tris pH 7.4	25	25	25	25
^{14}CIOA	30	30	30	30
dIH_2O	395	370	345	320
5-NO_2-Cyt	0	25	50	75

[S]=60μM

	I=0μM	I=10μM	I=20μM	I=30μM
Protein	50	50	50	50
Tris pH 7.4	25	25	25	25
^{14}CIOA	60	60	60	60
dIH_2O	365	340	315	290
5-NO_2-Cyt	0	25	50	75

[S]=100μM

	I=0μM	I=10μM	I=20μM	I=30μM
Protein	50	50	50	50
Tris pH 7.4	25	25	25	25
^{14}CIOA	100	100	100	100
diH_2O	325	300	275	250
5-NO_2-Cyt	0	25	50	75

[a]200 μM stock
[b]500 μM, 5000cpm/50μl
[c]all volumes in μl

Results and Discussion

Preliminary kinetic characterization of the enzyme IDCase seemed to be successful. The Michaelis-Menten constant estimated for IOA was acceptable with in the limits of statistical analysis. Data collected from four individual $^{14}CO_2$-displacement assays were averaged and a standard deviation calculated where K_m = 35 μM +/- 9. Refer to Figures 6-9 for Lineweaver Burk kinetic plots for individual K_m determinations.

The magnitude of this measurement agrees with that of thymine hydroxylase, overall K_m = 58 +/- 7. Consecutive enzymes in metabolic pathways are expected to have equivalent K_m values.

Kinetic parameters determined for the possible alternate substrate, 2-thio-iso-orotate presented a higher standard deviation. Data collected from five UV-vis spectrophotometric assays were averaged and a standard deviation calculated where K_m ~ 224 μM +/- 53. Because TIOA is not the original substrate of IDCase and therefore not as recognizable as IOA, this would explain the increased K_m for TIOA. The fact that IDCase recognizes TIOA as a substrate suggests that C2 of the heterocycle is not a site that is involved in ES complex formation, giving additional conformation of the proposed mechanism of IDCase.

Since both assays were attempted using impure protein, extraneous species might have interfered with enzyme-substrate interactions. Also, the amount of protein available for reaction and decreased specific activity may have introduced error. Data obtained using the $^{14}CO_2$-displacement assay with higher precision suggests that this technique is more sensitive and more accurate in determining kinetic parameters than the

spectrophotometric assay. Repeating the spectrophotometric assay upon obtaining purified protein would very likely increase precision of the data.

Mutant strain 2203 exhibited a 2-fold increase in IDCase specific activity than wild type strain 74A. This strain was used as a protein source for inhibition assays involving 5-nitrocytosine. Lines generated at [I] = 0 – 30 μM are closely linear with the most deviant R^2 value = 0.9864 (Figure 13a). The Lineweaver-Burk Inhibition plot (figure 13b) yielded K_i ~ 26 μM. K_i determination for 5-nitrocytosine indicates that this pyrimidine is a moderate inhibitor in comparison to 5-nitrouracil.

The attempts to directly measure the inhibition constant for 5-nitrouracil were unsuccessful. At a 20 nM 5-nitrouracil concentration, greater than 95% inhibition of enzyme activity was observed. The K_i was approximated using the Segel Plot[12] (Figure 5a), a value of [I]/Ki at this activity was extrapolated to ~ 40. Thus, the value of K_i ~ 0.5 nM. Although there exists significant error in this estimation, an order of magnitude for the inhibition constant has been established. Since the estimated K_i is low, it is not possible to measure the value accurately using Michaelis-Menten kinetics. For Michaelis-Menten kinetic conditions, [I] must be near the value of K_i, and [E] must be ~ 100 fold lower than [I]. [E] is probably not lower than ~ 1 nM in these assays.

The inhibition constant for the rare tautomer (see Figure 5b) form of 5-nitrocytosine was also estimated using the measured K_i and properties of the tautomer. This value, K_i ~ 2.5 nM, closely agrees with that of 5-nitrouracil, K_i ~ 0.5 nM. This suggests that the tautomeric form of 5-nitrocytosine could be responsible for inhibition activity [13].

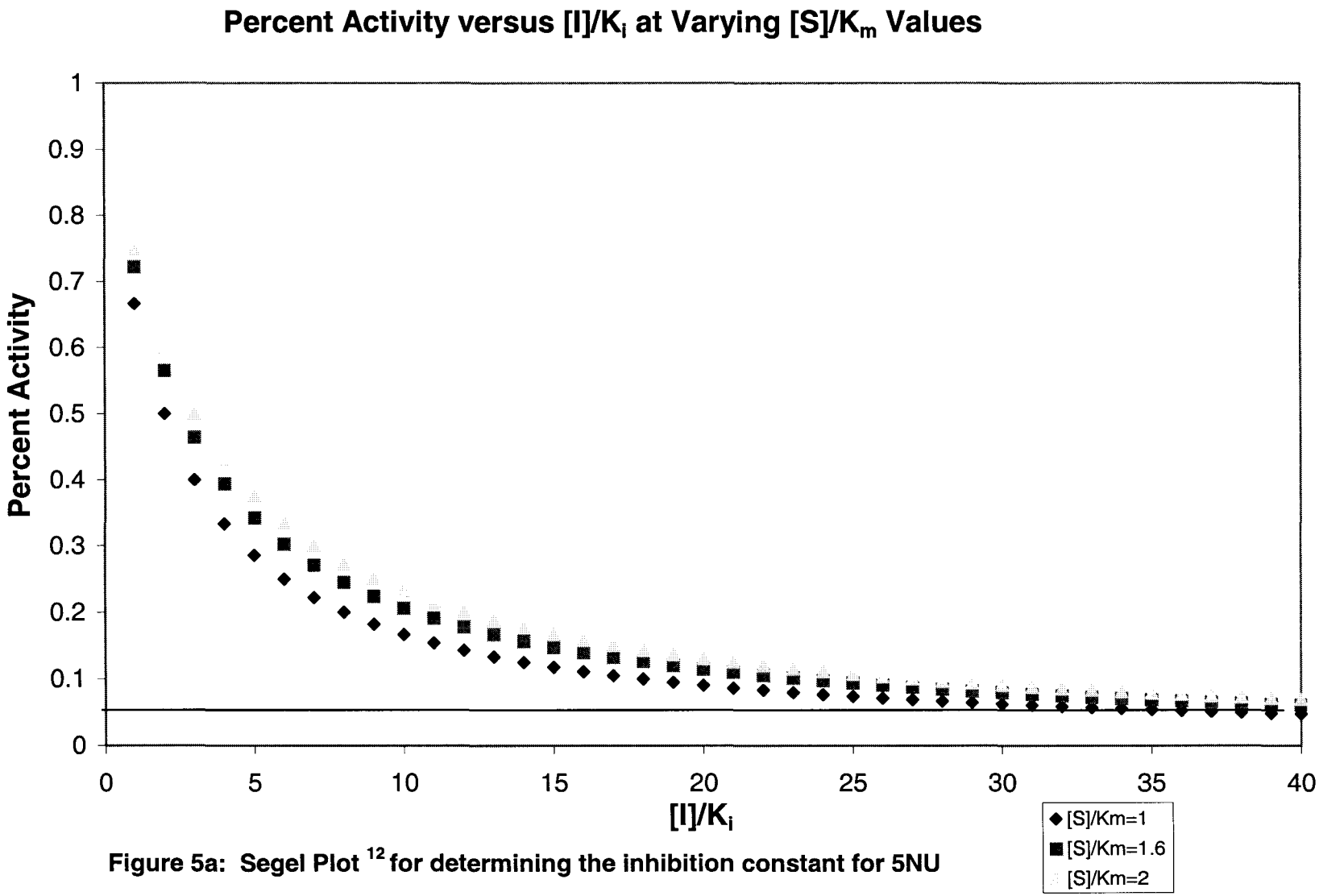
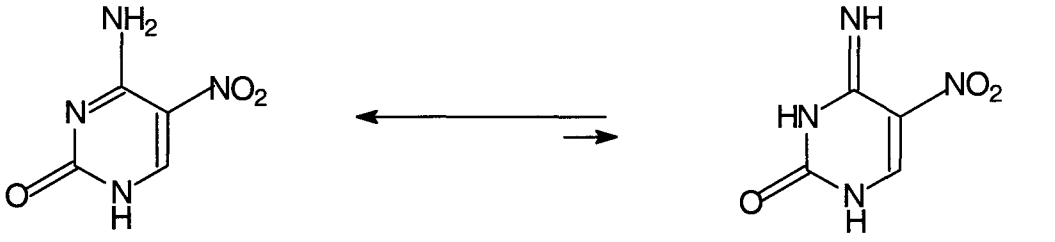


Figure 5a: Segel Plot [12] for determining the inhibition constant for 5NU



5-nitrocytosine
apparent K_i ~ 25 μM

5-nitrocytosine
intrinsic K_i ~ 2.5 μM

Figure 5b: Tautomeric shift of 5NC [13]. K_i approximated from Segal Plot in Figure 5a page 39.

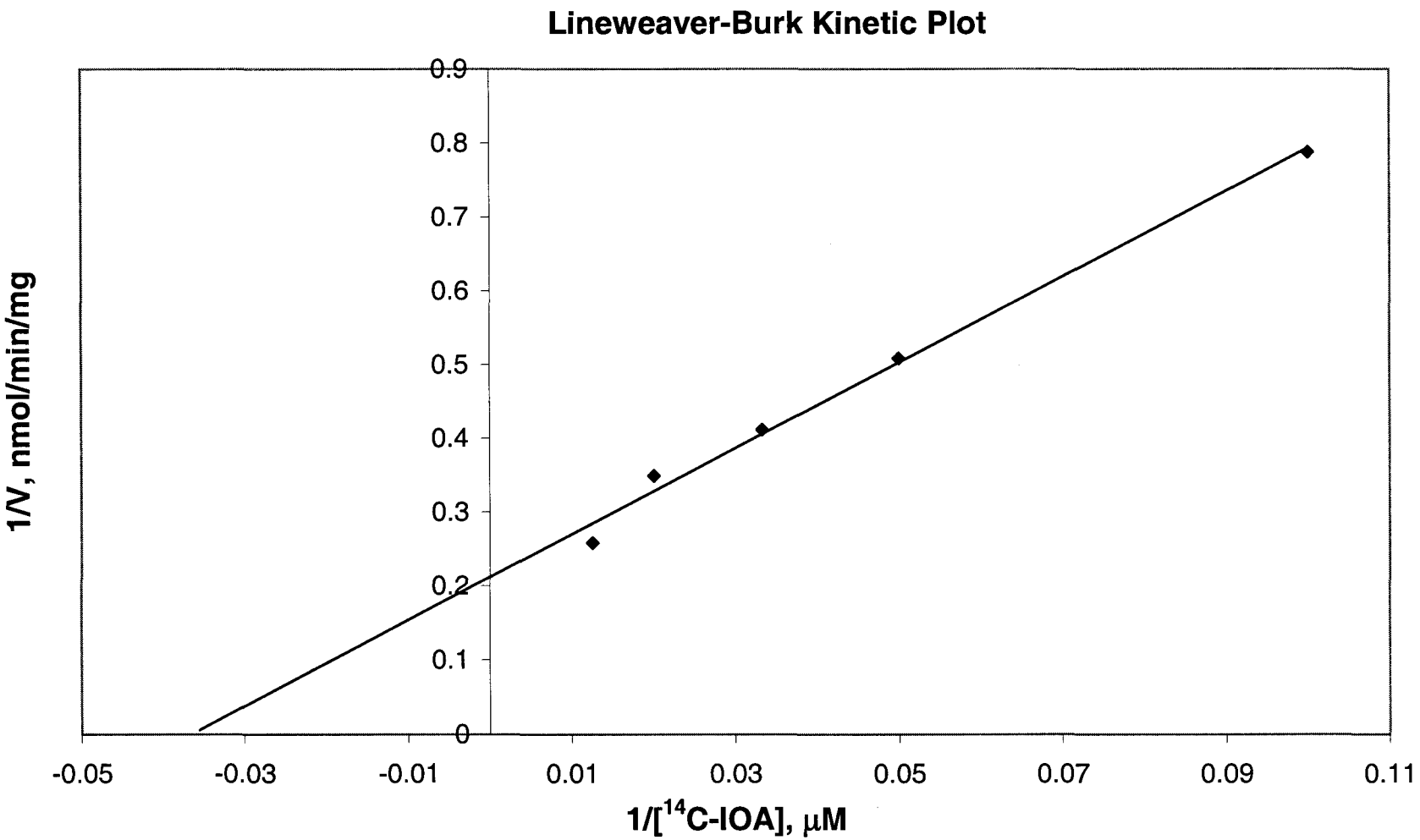


Figure 6: K_m determination for the original substrate, K_m ~ 27 mM. Average Km = 35 +/- 9

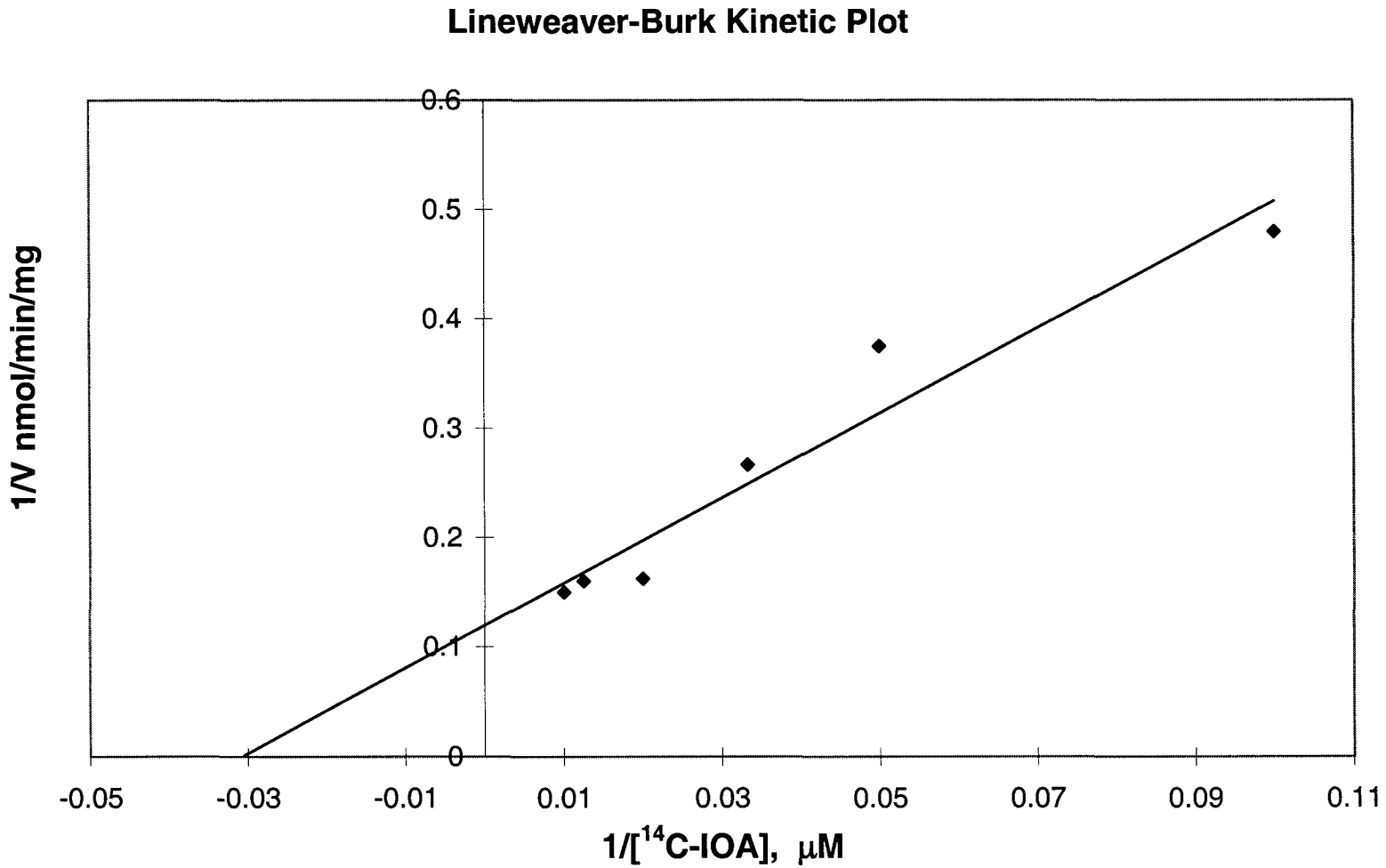


Figure 7: K_m ~ 42 μM, average K_m = 35 +/- 9

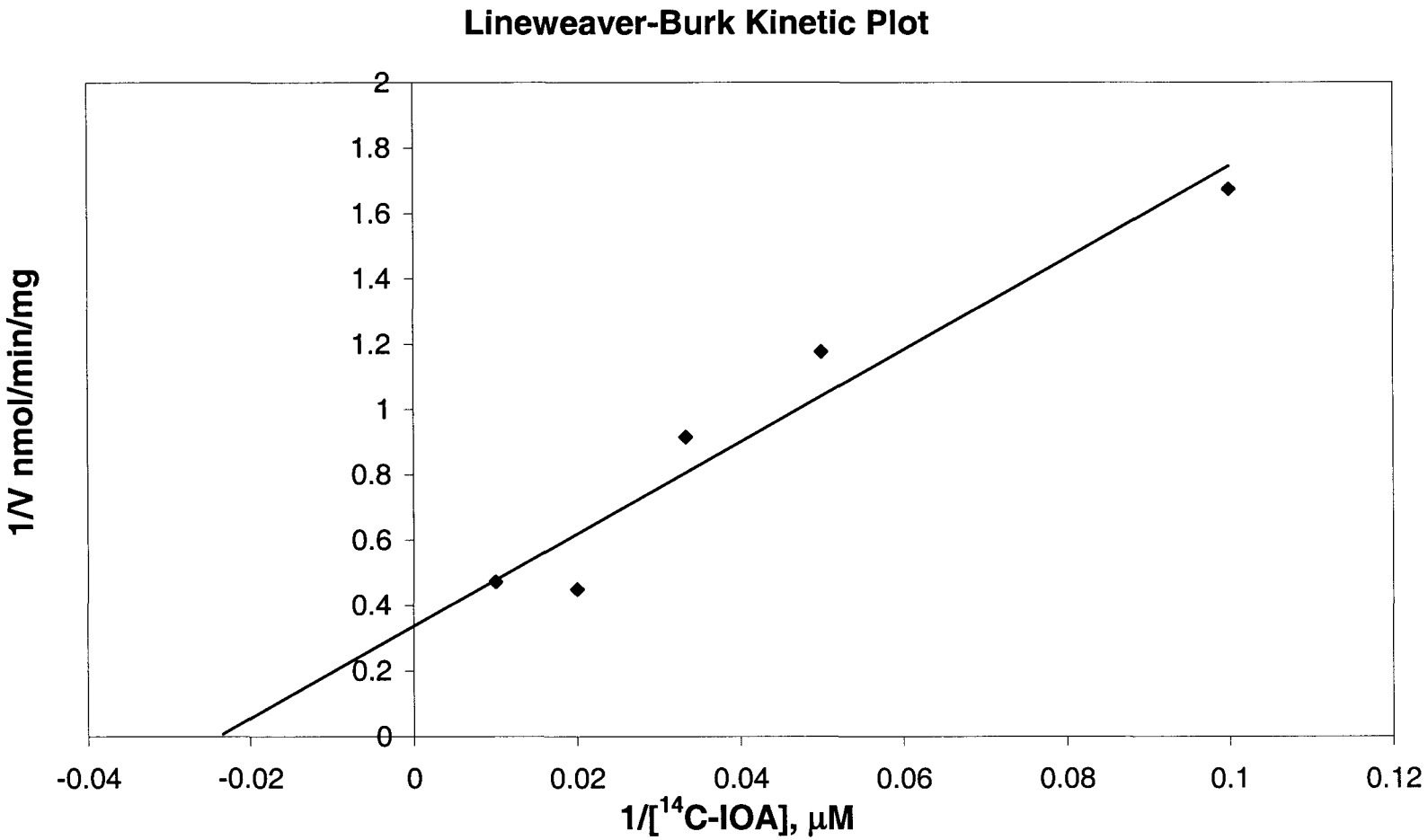


Figure 8: K_m ~ 42 μM, average Km = 35 +/- 9

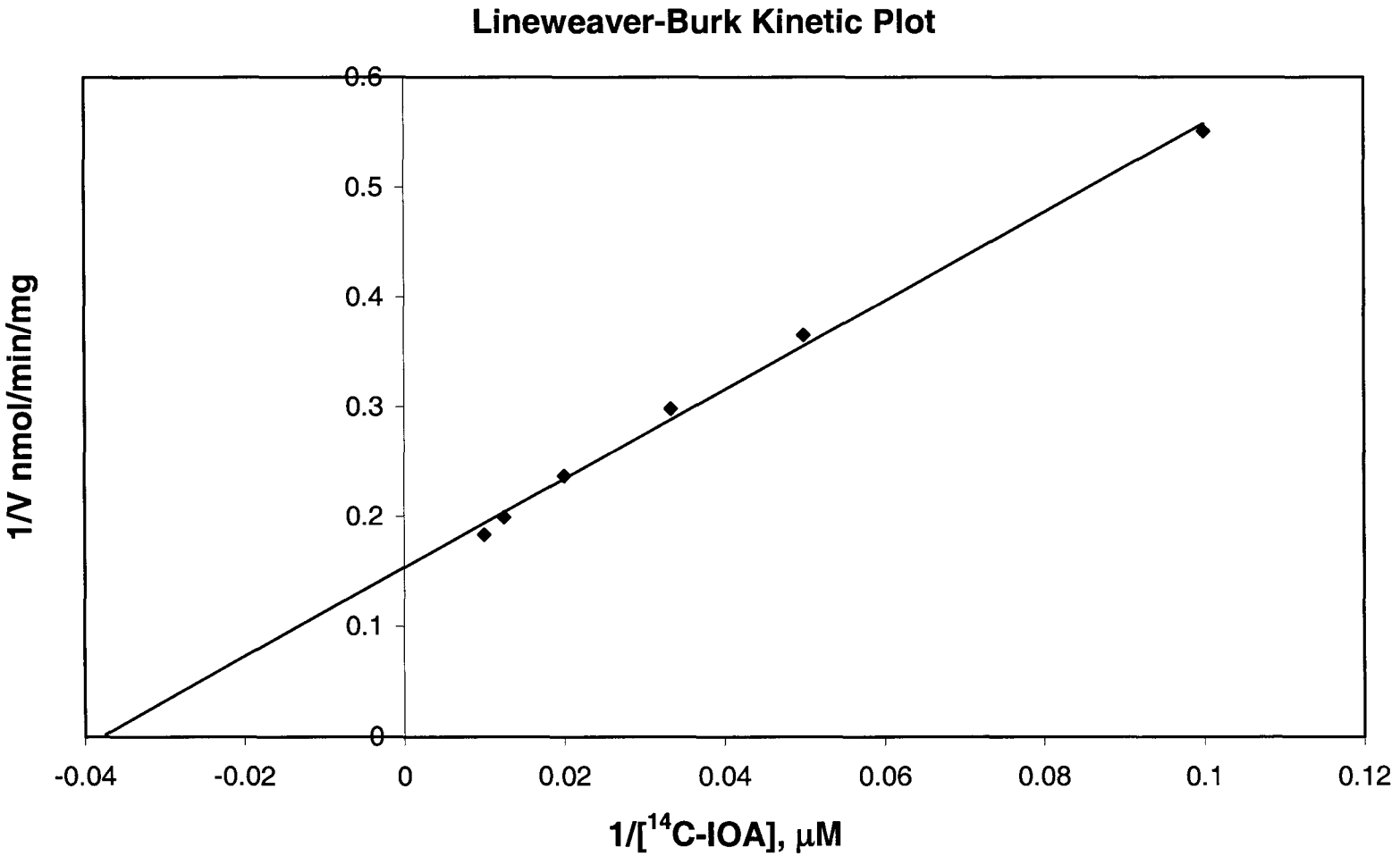


Figure 9: K_m ~ 26 μM, average Km = 35 +/- 9

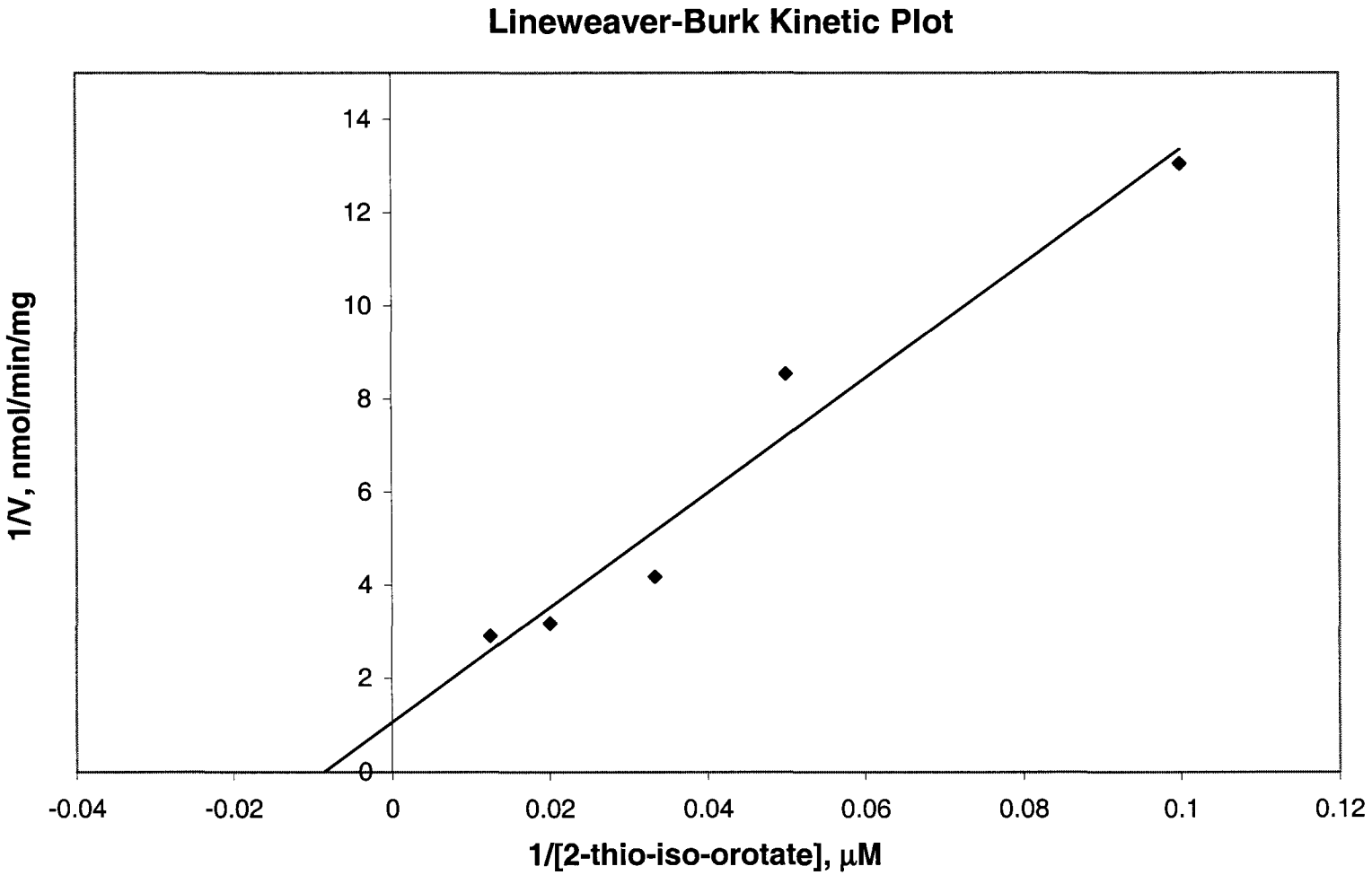


Figure 10: Km ~ 116 μM, average Km = 224 +/- 53

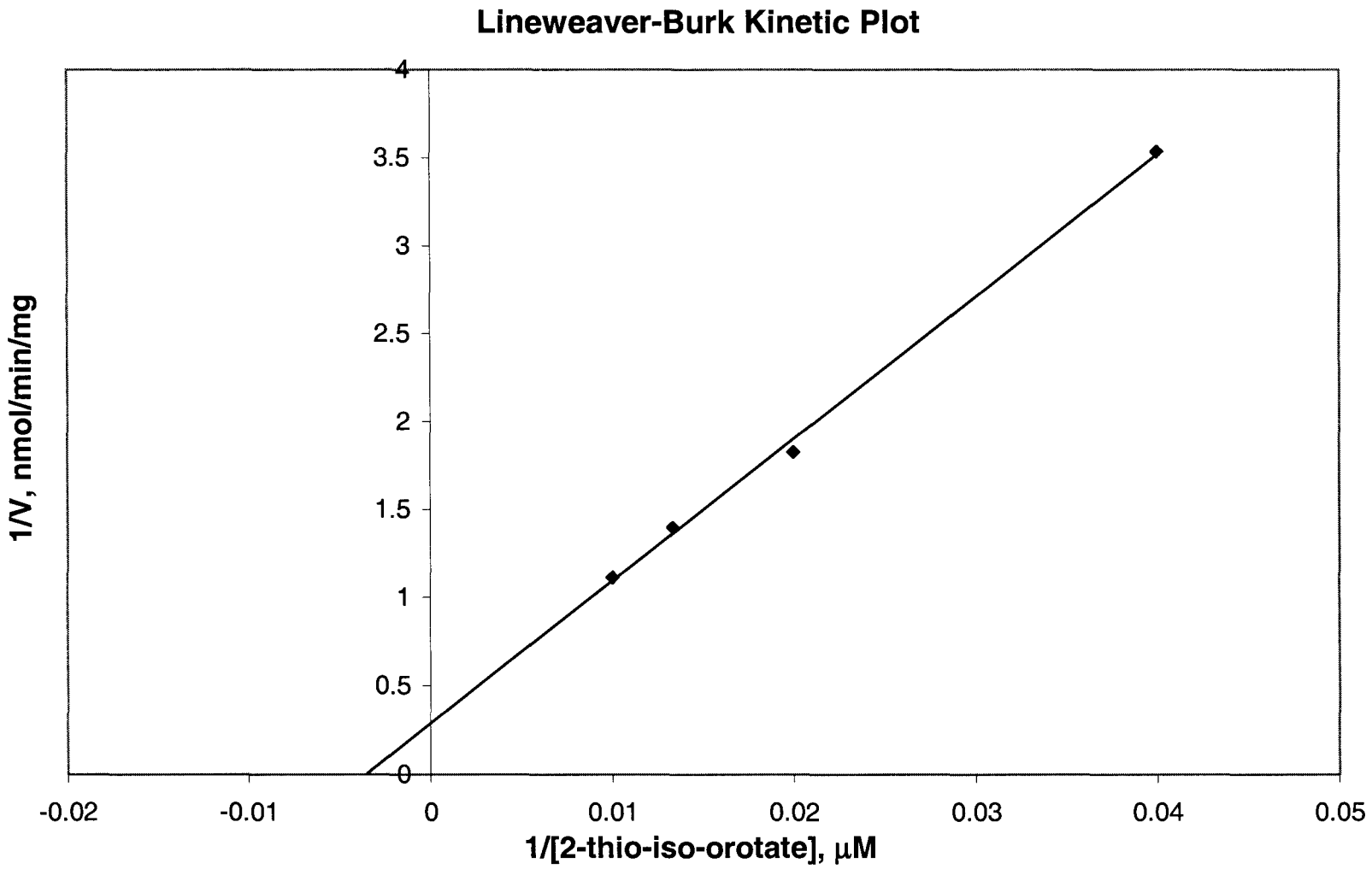


Figure 11: K_m ~ 280 μM, average K_m = 224 +/- 53

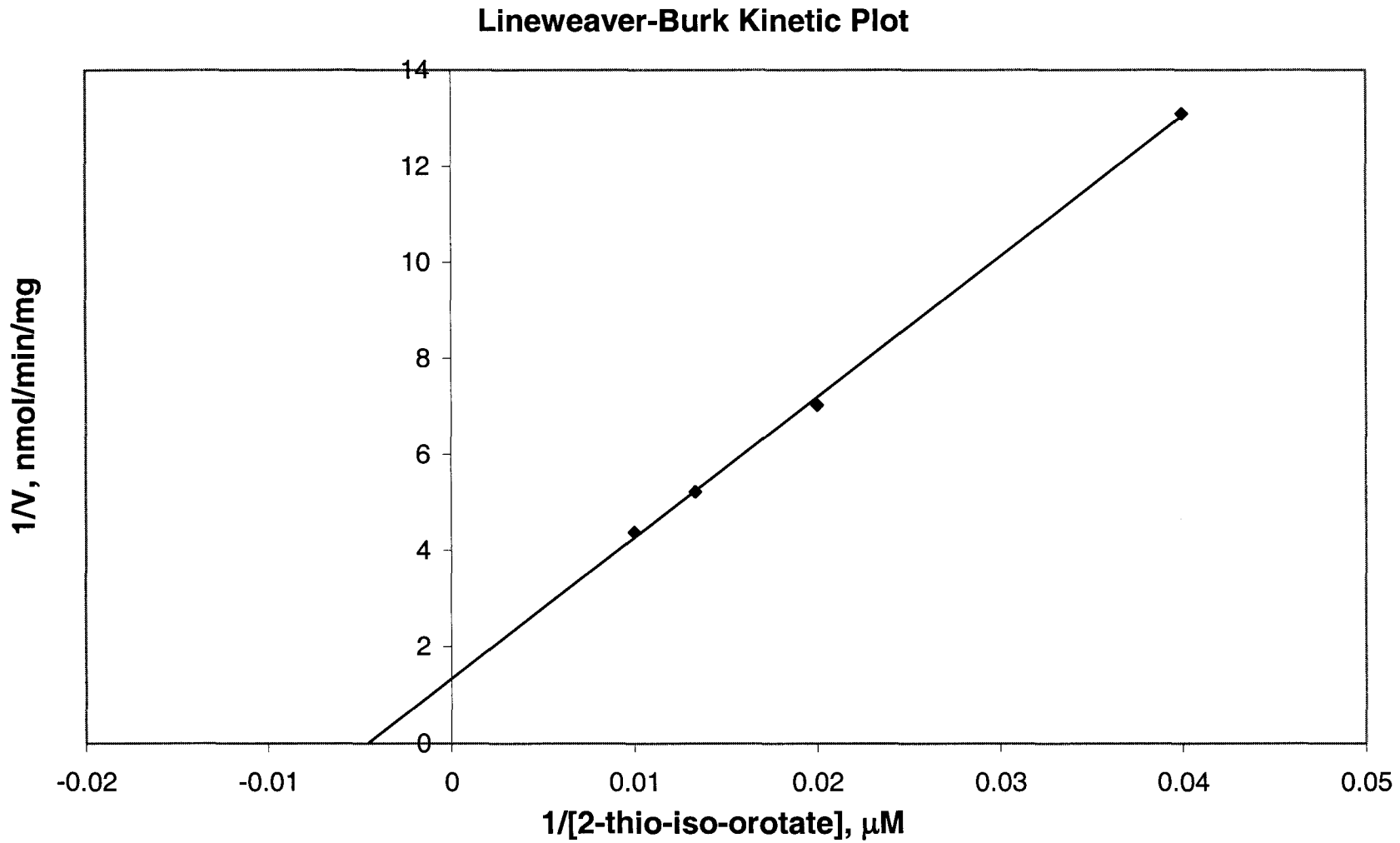


Figure 12: K_m ~ 218 μM, average K_m = 224 +/- 53

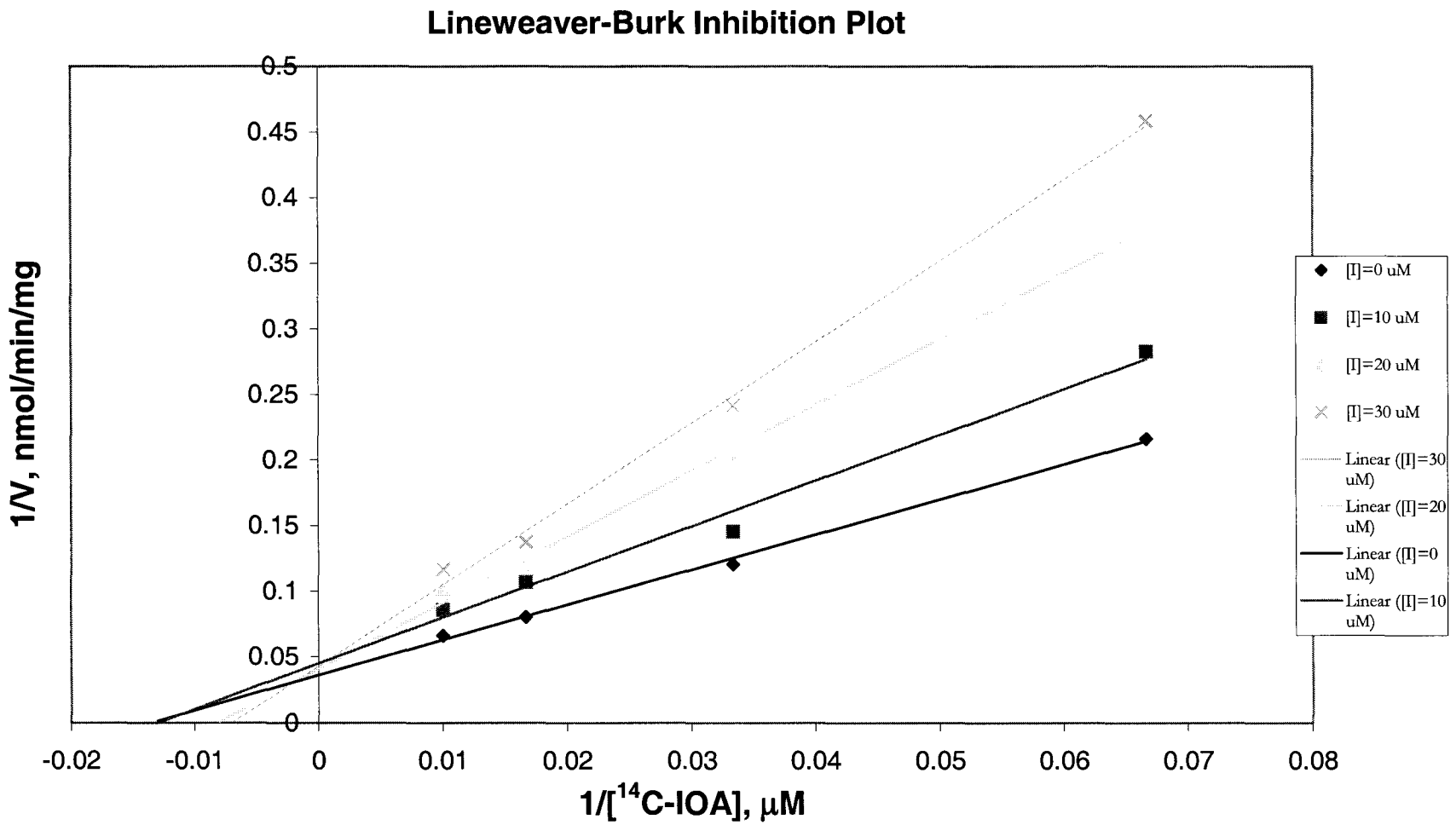


Figure 13a: K_m determinations at various concentrations of 5NC, resulting values of Km plotted in Figure 13b

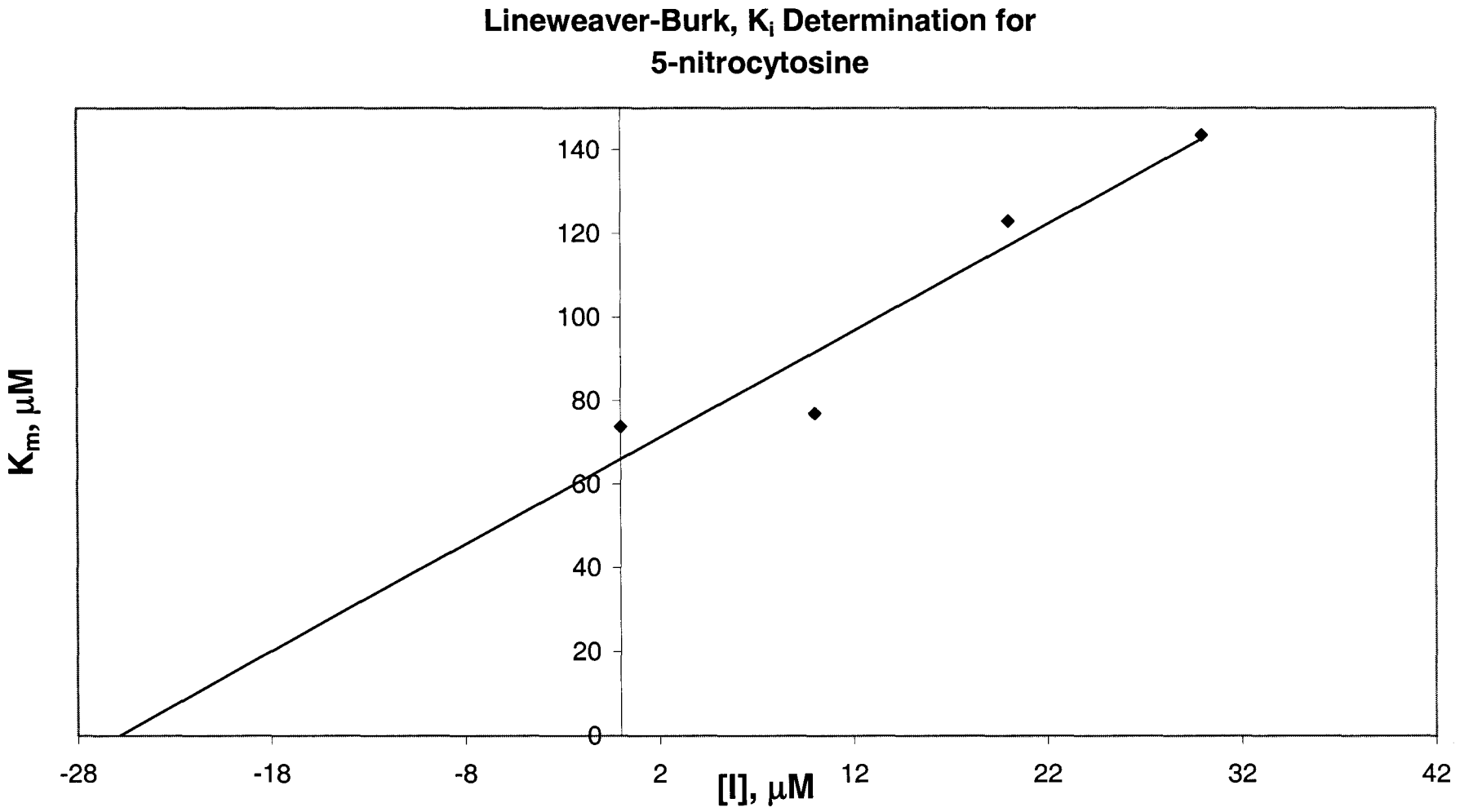


Figure 13b: K_i ~ 26 μM

Chapter 4 Purification

Attempted Purification of IDCase by Affinity Chromatography

Affinity chromotography was used in the attempted purification of IDCase. Columns from Amicon have been used separately in the purification of several enzymes in the citric acid cycle and the glycolysis pathway. Unlike other separation methods used, affinity chromatography utilizes specific binding sites of biological macromolecules. Separation occurs due to the binding ability of the protein of interest to a specific ligand, such as a substrate or inhibitor.

Synthetic dye ligand media has shown more beneficial to use in purification than other natural group selective media. For example, studies indicate the binding capacity of dye ligand media is greater than natural ligand media by a factor of 1:100 [14]. Because this media is resistant to enzymatic attack and degradation, it can be used for up to 40 runs over a two year period, making even large-scale purification more cost effective [15].

Dye ligand chromatography resins have been used in purifying several molecules of biological significance. Blue dextran, consisting of a high molecular weight dextran derivatized with triazinyl dye Cibacron Blue F3GA, was first used to purify pyruvate kinase in 1968 [16]. Haeckal *et al* also discovered the dye itself was responsible for protein binding, not the dextran. Blue dextran affinity ligand has been used in several experiments to purify yeast phosphofructokinase [17], various rat lactate dehydrogenases [18], and 3(17)-β-hydroxysteriod dehydrogenase from rat erythrocytres [19]. Hence, reactive blue 2 (referred to herein as blue A), Cibacron Blue F3GA (redesignated as 3GA), has been established as a groupselective affinity ligand, in native form or coupled to stationary support matrices [20].

Procion Red HE3B, reactive 120 (referred to as red A), has been shown to bind 30-60% of total protein. Enzymes such as carboxypeptidase G [17], NADPH dehydrogenases [14], malate dehydrogenase, lactate dehyrogenase, aldehyde reductase, and 5,10-methylenetetrahydofolate dehydrogenase have all been successfully purified using red A ligand [21,22].

Experiments conducted using dye ligands orange A, green A, and blue B have shown potential binding ability to proteins. Orange A is more selective than other dye ligands binding to pig heart citrate synthase and pigeon muscle carnitine acetyltransferase. However, only 5% of total protein bind to orange A. In contrast, green A binds nonselectively to many proteins. Lastly, blue B has selective binding capabilities similar to orange A. It has been sown to bind yeast phosphogycerate kinase and can be used to purify malate dehydrogenases [12].

In this experiment, five dye ligand columns were used to deterimine correct binding support of IDCase. A crude protein sample was applied to each medium, washed with buffer and fractionated. The resulting column fractions are analyzed by $^{14}CO_2$-displacement assays to determine if the protein had adhered to any of the columns.

Materials and Methods

Affinity Chromatography Dye Matrex sreening kit (Amicon) contained the following: five 9 x 32 mm polypropylene columns of various dye ligand matrices and one gel control all with 2 ml bed volumes. After initial regeneration step, all remaining steps were done at 4°C. Each column was washed with 12 ml of 8 M urea regeneration buffer. The columns were equilibrated in 12 ml of 20 mM Tris·Cl pH 7.5 starting buffer and allowed to drain to ~ 0.5 ml. 200 µl of IDCase ammonium fractionated lysate (resuspended pellet in 1.5 ml buffer I) was applied to each column along with 100 µl starting buffer. After a 30 minute "no flow" period, 10 ml of starting buffer was added and 1.5 ml fractions were collected manually. Any residual protein or other materials were eluted from the columns with 10 ml of elution buffer containing 20 mM Tris ·Cl pH 7.5 and 1.5 M KCl. A 2 ml bed volume of elution buffer was reserved and columns were stored at 4 °C until further use.

Analysis of Fractions using $^{14}CO_2$-displacement Assays A 450 µl sample from each column fraction was assayed to determine if any protein had adhered to the columns. 50 µl of reserved protein lysate was also assayed as a reference. Assay procedure has been described previously. Quenched reactions remained in acid ~8 hours until wicks were dried and counted.

Results and Discussion

Column fractions from five dye ligand chromatography matrices blue A, red A, orange A, green A, and blue B were analyzed for IDCase activity using radioassay techniques. The initial fractions from each column contained comparable IDCase activity to that of reference protein lysate. The cpm detected for gel control and all five dye ligand columns were very similar (within a 10 cpm range) suggesting that IDCase did not bind preferentially to any column. Thus, the dye ligands did not have the proper functional groups recognized by the enzyme active site.

Other types of affinity chromatography resins could possibly be used to purify IDCase. Synthesizing inhibitor analogs to be used as ligands is in progress (D.Yun Youngstown State University). The coupling of 5-nitrouracil through covalent linkage from N3 of the pyrimidine to an agarose support matrix has shown binding potential. Alkyl substitution at N3 of 5NU results in a compound with a moderate (~ 10-30 μM) inhibition constant. Substitutions made on the 5NU ring, including conversion to 5NC, allows for binding of the enzyme and elution before losing activity.

Chapter 5 Gene Isolation

Genetic Screening by Bacterial Complementation: *Escherichia Coli*

The specific gene encoding for IDCase protein has not been isolated from any cDNA library thus far. If isolated, the gene could be cloned and used to produce milligram amounts of protein. This would serve as a more convenient method of protein production for enzyme studies and purification.

Experiments attempting gene isolation were performed using a mutant strain of *E.coli* as a host organism. The general scheme of these experiments was to infect *E. coli* with foreign DNA (cDNA library containing genes showing IDCase activity) via a bacteriophage M13 cloning vector. Once incorporated into the cell, this vector can autonomously replicate itself, and be maintained as extrachromosomal DNA. Once infection has occurred, the bacterial cells are grown under varying conditions to test if the IDCase gene has been selected from the foreign cDNA library.

As with all other bacteria, *E.coli* has not been shown to contain the thymine salvage pathway and thus lacks the IDCase gene in its genome. Mutant strain JFS116 is lacking the ability to produce UMP by interupting the gene which encodes for ODCase. These cells grow only in the presence of uracil, which is converted to UMP by the enzyme UPRTase. If the bacterial cells of this strain were starved of uracil, it is possible in this experiment that IOA could serve as the sole pyrimidine source if the cells acquired the IDCase gene from the screening library. This allows for simple detection of colonies with IDCase expression. If the plasmids pick up a gene other than the one encoding for IDCase, the cells would not grow on (-) pyrimidine media.

However, colonies that grow on media containing IOA (-) URA must also be tested for the gene that encodes for ODCase. In the *de novo* pathway ODCase catalyzes the conversion of OMP to UMP, which is directly incorporated in DNA and RNA. Therefore, uracil is not needed as a pyrimidine source and colonies appearing on IOA (-) URA must be differentiated between IDCase and ODCase gene expression.

Plasmid DNA from colonies, which may contain the gene, was separated from bacterial DNA using plasmid preparation techniques [23] and reintroduced into JFS116 cells treated with calcium chloride [24]. This allows for transformation of plasmid DNA into a new sample of host bacteria by increasing the permeability of cells to small DNA molecules. These cultures, which contain ~1/10,000 competent transformants, are transferred to restrictive media to test for the presence of the plasmids containing the IDCase gene.

RecA$^-$ JFS116 was used as a host organism for all screening experiments. This strain contains an interruption on the ODCase gene with an antibiotic resistant insert. Colonies were grown on (+) antibiotics media to ensure the marker was still in place.

Materials and Methods

Media Preparation for IDCase Gene Screening Experiments

All chemicals were purchased froms standard sources. Complete Minimal Media (CMM) was prepared in 100 ml batches containing 80 ml diH_2O water, 10 ml M9 salts, and 2.5 mg D,L tryptophan. Bactoagar (1.5 g) (Fisherbiotech) was added just before sterilization (Uamato SM 32). After slightly cooling the media to ~60 °C, 10 ml of 10% filter sterilized glycerol was added along with 100 µl of the following: 1M $MgSO_4$, 0.1M $CaCl_2$, 1mg/ml thiamine, and 10mg/ml tetracycline (dissolved in ethanol). 60 µl of 50mg/ml kanamyacin was also added to the mix.

Approximately 25 ml of CMM was aliquoted to Fisher brand polystyrene Petri plates (size 100 x 15mm) with the following additives in a ratio of 1 µl per ml of media. For positive control plates, 50mg/ml ampacillin and 20 mg/ml uracil was added. To screen for the plasmid containing the IDCase gene, µl 50mg/ml ampicillin and 20mg/ml IOA was added. To differentiate between IDCase and ODCase selection, plates containing only 100 µl 50mg/ml ampacillin were prepared. All plates were vertically positioned to minimize condensation and immediately used after cooling or stored at room temperature for less than 24 hours.

Phage Infection of *Echerichia Coli* *E. coli* mutant strain JFS116 was stored in 20% glycerol at -70°C until use. A sterile borosilicate culture tube (Fisher) containing 2 ml L. Broth (LB, Table 8) was inoculated with a small portion of JFS116. The frozen bacterium was abstracted using a sterile toothpick and immersed into the LB. The culture was incubated at 37 °C with shaking in a dry type bacterialogical incubator for 1 hour. 2 µl each 30mg/ml kanamycin and 10 mg/ml tetracycline was added followed by continued incubation for an average of 12 hours. After this time, the cell suspension was diluted 1/100 with fresh LB (usually 40 µl of suspension and 4 ml LB) and previous amounts of antibiotics. Continued incubation with vigorous shaking followed for an average of 3 hours until OD_{600} = 0.3-0.4 was reached. This is indicative of a cell density of ~ $5x10^7$ cells. Cell concentration was estimated using a Bausch & Lomb Spectronic 20 spectrophotometer by measuring the percentage transmittance of 0.5 ml cell culture diluted with 4.5 ml LB. Absorbance was calculated from A = 2-log%T and corrected for dilution. The average OD_{600} used from cultures at this stage of growth was 0.45. At this time 1.5 ml of cell suspension was transferred to a sterile tube and infected with 8 µl of phage solution which corresponds to ~ $8x10^4$ phage. Infected cell suspension was incubated under anaerobic conditions for an additional 15 minutes. Cell collection was done by a 5minute centrifugation at 4000 rpm and resuspended in 1.2 ml CMM alone. 50 µl aliquots were spread with a flame-sterilized spreader to 25 CMM plates containing Amp and IOA. One 50 µl aliquot was spread on one positive control plate containing Amp and Ura. Following a 3 day incubation at 37 °C, colonies appearing on CMM + Amp + IOA were tested for the plasmid.

Plasmid Preparation: Lysis by Alkali Colonies from 5 CMM + Amp + IOA plates were transferred with a sterile toothpick to separate tubes containing 2 ml L. Broth for 1 hour with vigorous shaking at 37 °C. After this time 2 µl 50 mg/ml Amp was added with continued incubation for ~12 hours. Cell suspensions from each were spun at 4000 rpm for 10 minutes and corresponding bacterial pellets were resuspended in 200 µl of solution I (Table 8). The suspensions were transferred to sterile eppendorf tubes and remained at room temperature for 5 minutes. Upon addition of 400 µl solution II (Table 8) suspensions were mixed by inversion and remained on ice for 10 minutes. An additional 10 minute ice incubation followed a 300 µl addition of a neutralizing solution (Table 8). Colloidal suspensions were centrifuged at 14,000 rpm for 30 minutes using Eppendorf centrifuge 5415C. The cell debris was discarded and 750 µl of supernatant from each sample was mixed with 450 µl isopropanol (Fisher) in sterile Eppendorf tubes. After a 15-minute incubation period at room temperature, the plasmid DNA was recovered by centrifugation at 14,000 rpm for 30 minutes. Plasmid pellets were triple washed with 70% ETOH and resuspended in 100 µl of TE buffer pH 8.0 (Table 8). Th plasmids remained on ice for ~ 3 hours before use in the calcium chloride transformation procedure.

Reintroduction of Plasmid DNA into *E.Coli* strain JFS116 by $CaCl_2$ Transformation

An overnight culture of uninfected JFS116 from –70 °C freezer stock was started in 2 ml of LB as previously described. To a 250 ml Erlenmeyer flask 1 mL of culture was used to inoculate 50 ml LB containing 50 µl each of 30 mg/ml kanamycin and 10 mg/ml tetracycline. Following an incubation period of 3 hours with vigorous shaking at 37 °C, the cell suspension was chilled on ice for ~ 10 minutes. The cell pellet was obtained by centrifugation at 4000 rpm for 5 minutes at 4 °C. The pellet was resuspended in ice-cold 25 ml $CaCl_2$ solution (Table 8) and placed in ice for 15 minutes. The pellet was collected by centrifugation at 4000 rpm for 5 minutes at 4 °C. The cells were resuspended in 3 ml of ice-cold $CaCl_2$ solution and mixed separately with DNA plasmid 1-5 (preparation described previously). The mixture was left on ice for 30 minutes. The cells were then heat shocked in a 42 °C water bath for 2 minutes and immediately placed in an ice bath for 10 minutes. The cells were then transferred to sterile polystyrene tubes containing 1 ml LB and incubated for 1 hour at 37 °C without shaking. A flame sterilized loop was used trransfer cells from each tube were spread on individual agar plates containing 25 ml LB with 100 µl each of 30 mg/ml kanamycin, 10 mg/ml tetracycline, and 50 mg/ml ampicillin. Transformed colonies, ~ 100/plate, appeared within 24 hours of incubation at 37 °C.

Table 8: Solutions for IDCase Gene screening Experiments

Description	Conc. of Reagents (Fisher)	Preparation Summary
L.Broth	tryptone yeast extract NaCl	10g tryptone, 5g yeast extract, and 10g NaCl dissolved in 500 ml dIH_2O pH 7.4 with 6M NaOH, autoclaved
Solution I	50 mM glucose 25 mM Tris·Cl pH 8.0 10 mM EDTA	2.2ml 20% glucose, 1.25mL 1M Tris, 1.0ml 0.5M EDTA Prepared in 50 ml portions and
Solution II	0.2M NaOH 1% sodium dodecyl sulfate (sigma)	0.5ml 10% SDS solution was added to 1.0ml 1M NaOH and diluted to 5.0ml with dIH_2O water
Neutralizing solution	5M potassium acetate pH glacial acetic acid	49.07g $KC_2H_3O_2$ mixed with 100 ml diH_2O, pH adjusted to 8.0 with 6M Mixed 6.0ml with 1.15 conc. glacial acid and 2.85ml diH_2O
1X TE Buffer	10 mM Tris pH 8.0 1 mM EDTA pH 8.0	Prepared 100X stock by dissolving 60.57g Tris and 1.86g EDTA in 500mL dIH_2O. 100µl 100X diluted to 10 ml
Transformation	50 mM $CaCl_2$ 10 mM Tris·Cl pH 8.0	125ml 1M Tris (121.14g dissolved in added to 0.25ml 1M $CaCl_2$ and diluted up to 25 ml with diH_2O, autoclaved in two 25ml portions

IDCase/ODCase Selection Experiment

With a flame sterilized loop, transformant colonies from individual LB + Kan + Tet + Amp plates were transferred to the following: CMM + 100 µl IOA + 100 µl Amp, CMM + 100 µl URA + 100 µl Amp, and CMM + 100 µl Amp only. Plates were inverted and incubated at 37 °C in a gravity convection incubator (Precision). Colonies did not appear in 18 hours, 24 hours, or 48 hours.

IDCase/ODCase Activity Experiment

In this experimnet, bacterial cell lysates from colonies picked in the sreening experiment were assayed for IDCase and ODCase. Transformant colonies from LB + Kan + Tet + Amp plates used in the previous experiment were transferred with a sterile toothpick to flasks 1-5 containing 25 ml sterile LB and 100 µl 50 mg/ml ampicillin. Controls for ODCase activity were also prepared using frozen stock (-70°C) JM101 (+) and JFS116 (-). Both strains were transferred using a sterile toothpick in flasks 6 and 7 containing 25 mL sterile LB. 100 µl of 30 mg/ml kanamycin and 10 mg/ml tetracycline (in ethanol) were added to JFS116. Cultures were incubated at 37°C with vigorous shaking for ~24 hours after which time a dense cell suspension appeared. Cell suspensions were subjected to sonication (Branson Cell Disruptor model 185) for three 10 second bursts with intermittent 30 second ice treatments and centrifuged at 4000 rpm for 5 minutes at 4 °C. Bacterial lysates were assayed using the same method in determining IDCase activity in *N. crassa* lysates. To test for IDCase activity, 50 µl of 500 µM (5000cpm/50µl) [14-C]iso-orotate was mixed with 450 µl bacterial lysate from flasks 1-5. The reactions were allowed to incubate for 10-minute time periods at 37°C with vigorous shaking. To determine if ODCase activity was present, 50 µl of 25 mM

(2500cpm/50μl) [7-^{14}C]OMP was incubated with 450 μl bacterial lysate from 1-5. 450 μl of bacterial lysate from 6 (JFS116) and 7 (JM101) was reacted with 50 μl of ODCase and IDCase substrates for a negative and positive controls.

Results and Discussion

Attempts to isolate the gene which encodes for IDCase have been unsuccessful. The plasmids from colonies initially growing on restrictive IOA (+) antibiotic media, which were thought to have picked up the IDCase gene from a cDNA library via phage infection, were isolated and transformed into a new JFS116 culture. The final step in these experiments, which involved growing transformed colonies on restrictive media containing IOA and antibiotics only, yielded a negative result. There are two explanations for this. Cells could not use IOA as its only pyrimidine source indicating the IDCase gene was not picked, but the ODCase gene was. Minimal counts resulting from radioassays to detect IDCase/ODCase activity support absence of the IDCase gene, but do not support the presence of the ODCase gene. Furthermore, the second possibility that the interruption of the ODCase gene has been repaired resulting in ODCase expression, cannot be supported. If the interruption of the ODCase gene had been repaired then the antibiotic resistant insert would also have been cut out. Without an antibiotic resistant gene, these colonies would not have survived on media containing antibiotics. In conclusion, the gene expressing ODCase could have been picked from the cDNA library but the concentration of this enzyme in the cells might have been too low for immediate detection in the radioassay. Repeating this assay with an increased incubation period or assaying a larger number of colonies may give additional information. Attempts at isolation of the IDCase gene are ongoing.

Conclusion

The radioactivity-based assay used to measure kinetic parameters for IDCase has proven to be a precise quantitation method in determining activity of this enzyme. The standard deviation corresponding to Michaelis-Menten constants measured using this technique were quite acceptable given that impure protein was used. The inhibition constant measured for 5-nitrocytosine is reasonable when compared to 5NU. Lineweaver Burk inhibition data used to calculate the K_i agrees with the theoretical representation of a competitive inhibitor. All lines are closely linear (R^2 values within range 0.9951-0.9973) with the exception of the most deviant line having an R^2 value = 0.9874. Extraneous species in crude protein solution could be responsible for errors. All experiments should be repeated once purified protein is achieved.

Purification experiments have yielded definite negative results. Several commercially available dye ligands used in affinity chromatography experiments do not have functional groups recognized by IDCase, or these functional groups are too large to fit in the active site of the protein. Syntheses of inhibitor analogs to be used as affinity ligands in further purification attempts are ongoing.

The attempts to isolate the gene encoding for IDCase from cDNA libraries were unsuccessful. Isolation via bacterial complementation techniques could possibly be done with repeated trials. Repeated experiments using electrophoresis as a verification method in addition to the radioactivity-based assay are ongoing. Isolation and eventual cloning of the gene would provide access to large amounts of enzyme for enzymological studies.

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