THE ENZYMES OF NITRATE ASSIMILATION IN *SCLEROTINIA SCLEROTIORUM*

by

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PREFACE

Part of the work described in this thesis has been presented at the Australian Biochemical Society Conference (Canberra, 1985) and published in the following journals:

1. Some properties of glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*.
   M.A. Rachim and D.J.D. Nicholas (1985)
   Proc. Aust. Biochem. Soc. 17, 21

2. Glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*.
   M.A. Rachim and D.J.D. Nicholas (1985)
   Phytochemistry 24, 2541-2548

3. Some properties of nitrate reductase from *Sclerotinia sclerotiorum*.
   M.A. Rachim and D.J.D. Nicholas (1986)
   Phytochemistry 25 (in press)

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief no material described herein has been previously published or written by another person except when due reference is made in the text.

If accepted for the award of a M.Ag.Sc. degree, this thesis will be available for loan and photocopying.

M.A. RACHIM

ABBREVIATIONS

The abbreviations for chemicals and symbols in general follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem. J.* (1966) 101: 1-7) or the Instruction to Authors for the Phytochemistry (*Phytochemistry* (1983) 22: 1-7).

CHEMICALS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
BV	Benzyl viologen
BVH	Benzyl viologen (reduced)
CDP	Cytidine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
CMP	Cytidine 5'-triphosphate
Cyt b_{557}	Cytochrome b_{557}
DCPIP	2,6-Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethyl cellulose
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide (reduced)
FMN	Flavin mononucleotide
$FMNH_2$	Flavin mononucleotide (reduced)
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
IDP	Inosine 5'-diphosphate
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
MSX	L-methionine-DL-Sulphoximine
MV	Methyl viologen
MVH	Methyl viologen (reduced)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide
p-CMB	p-chloromercuribenzoate
SDS	Sodium dodecyl sulphate
-SH	Sulphydryl group
SVD	Snake venom phosphodiesterase
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine 5'-diphosphate
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate

Symbols and Units

A	absorbance
°C	degree Celcius (centigrade)
cm	centimeter
g	gram
g	unit of gravitational field
hr	hour(s)
kDa	kilo dalton(s)
K_i	inhibitor constant
K_m	Michaelis-Menten constant
L	litre
M	molar
mA	milliampere
mg	milligram
min	minute(s)
ml	millilitre
mm	millimeter
mM	millimolar
mmole	millimole(s)
MW	molecular weight
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
µmole	micromole(s)
N	normal
nm	nanometer
nmole	nanomole(s)
%	percent
P_i	inorganic phosphate
p.s.i.	pound per square inch
U.V.	ultra violet
v	volume
w	weight

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SUMMARY

1. This thesis embodies results of an investigation on some biochemical aspects of enzymes involved in nitrate assimilation in a plant-pathogenic fungus, *Sclerotinia sclerotiorum*. The enzymes are nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthase.

2. Nitrate reductase, purified 118-fold, had a molecular weight of 210 kDa and was composed of 2 dissimilar subunits of 123 and 107 kDa. In addition to using NADPH as an electron donor, the enzyme also utilized reduced viologen dyes and reduced flavin nucleotides as reductants.

3. FAD was isolated from the purified enzyme; however, exogenous FAD was required for maximal activity of NADPH-dependent nitrate reductase *in vitro*. FMN did not substitute for FAD.

4. The enzyme activity was inhibited by metal chelating agents and by flavin and sulphydryl group inhibitors. Azide markedly restricted both NADPH- and MVH-dependent reactions, but inhibition by p-CMB, NEM and amytal was more marked when NADPH was the reductant.

5. Nitrite inhibited nitrate competitively in NADPH-dependent nitrate reduction *in vitro*; however, it is unlikely to inhibit nitrate reductase activity under physiological conditions because the K_i for nitrite was three-fold greater than the K_m for nitrate.

6. Nitrite reductase, the second enzyme in the nitrate assimilation pathway utilized NAD(P)H, reduced viologen dyes and reduced flavin nucleotides as electron donors. NADPH was the most effective electron donor. Maximal activity of the NADPH-dependent nitrite reductase

was achieved by adding FAD to the assay mixture; FMN however, was less effective.

7. Associated with the nitrite reductase enzyme was a hydroxylamine reductase activity as well as diaphorase type activities utilizing either ferricyanide, DCPIP or cytochrome *c* as an electron acceptor.

8. The product of both nitrite and hydroxylamine reductases was ammonia but hydroxylamine was not an intermediate product of nitrite reductase. The stoichiometry of NADPH oxidized to nitrite utilized and ammonia produced in NADPH-dependent nitrite reduction was 3:1:1 and the ratio of NADPH oxidized to ammonia formed in the hydroxylamine reductase-mediated reaction was 1:1.

9. In the presence of FAD, nitrite reductase was inactivated by preincubation with NADPH, but $NADP^+$ was without effect. This inactivation was offset by the substrates (nitrite and hydroxylamine).

10. Nitrite reductase was sensitive to metal binding agents as well as flavin- and sulphydryl group-inihibitors. The inhibition by p-CMB and mepacrine respectively, were reversed by adding cysteine and FAD.

11. Washed felts readily incorporated $^{15}NH_4^+$ into cell-nitrogen but this effect was inhibited by both MSX and azaserine, inhibitors of glutamine synthetase and glutamate synthase respectively. Since glutamate dehydrogenase was not detected in cell-free preparations, this is further evidence that the glutamine synthetase/glutamate synthase pathway is the main route for the assimilation of ammonia into amino acids.

12. Glutamine synthetase, purified by ion exchange and affinity chromatography, had a molecular weight of 490 kDa. The enzyme was composed of 8 identical subunits of 60 kDa. The transferase activity of the

enzyme required Mn^{2+} and ADP for maximal activity whereas the biosynthetic activity required Mg^{2+} and ATP.

13. The enzyme was regulated by feedback inhibition involving amino acids and organic acids and by a adenylylation/deadenylylation mechanism. The transferase activity of the enzyme was also inhibited competitively by the substrates of the biosynthetic reaction, namely glutamate and NH_4Cl, with respect to glutamine.

14. Glutamate synthase had a molecular weight of 220 kDa and was composed of 4 subunits of 53.7 kDa. The enzyme had a specific requirement for NADPH, α-ketoglutarate and glutamine as the electron donor, amino acceptor and amino donor, respectively.

15. Glutamate synthase was regulated by feedback inhibitors including amino acids, organic acids and nucleotides. The enzyme was markedly inhibited by p-CMB (this effect was reversed by cysteine), O-phenanthroline, α,α'-dipyridyl and azaserine.