

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

THE INTRACELLULAR SITE OF SYNTHESIS OF FRUCTOSE 1,6-BISPHOSPHATASE  
IN RAT LIVER

A thesis presented in partial fulfilment of the requirements  
for the degree of Doctor of Philosophy in Biochemistry at  
MASSEY UNIVERSITY

JOHN WILLIAM McLEAN  
1979

## ABSTRACT

Fructose-1,6-bisphosphatase (FBPase, E.C. 3.1.3.11) has been purified from rat liver cytoplasm by a new purification procedure. Monospecific antibodies were raised to FBPase in rabbits to enable the immunochemical isolation and quantitation of FBPase in protein homogenates.

Pulse labelling of rat liver in vivo showed that the synthesis of FBPase amounted to 0.89% of total soluble protein synthesis. Newly synthesized FBPase was found almost entirely in liver cytoplasm in contrast to serum albumin which was associated only with microsomes, and amounted to 16.7% of total microsomal protein synthesis.

Isolated free and bound polysomes from liver synthesized almost equal amounts of FBPase when incubated in vitro, whereas albumin synthesis was confined to bound polysomes. Premature termination of translation led to the release of partial protein transcripts which were specifically immunoprecipitated.

Affinity-purified  $^{125}\text{I}$ -labelled antibodies to FBPase and albumin were used to quantitate nascent protein chains on free and bound polysomes. FBPase antibody bound to both classes of polysome with almost equal affinity but albumin antibody was only associated with bound polysomes.

Poly(A)<sup>+</sup> RNA isolated from free and bound polysomes by poly(U)-Sepharose chromatography was translated in a cell-free protein-synthesizing system derived from wheat germ. RNA from both classes of polysome synthesized FBPase, but albumin was only synthesized in response to bound polysomal RNA. Some full length transcripts corresponding to both proteins were produced in wheat germ extracts, but a large amount of smaller molecular weight material was specifically immunoprecipitated. Total translation products from both classes of polysome also produced considerable abnormally short molecular weight material, but only full length transcripts were produced when the system was programmed with TMV RNA.

Since liver cells contain twice as many bound polysomes as free, it has been concluded that about 70% of intracellular FBPase synthesis takes place on bound polysomes. In contrast, albumin synthesis was almost totally confined (>97%) to bound polysomes and newly synthesized

albumin was segregated into microsomes. The partitioning of synthetic activity for albumin is therefore a result of the distribution of mRNA coding for this protein.

## ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr John W. Tweedie for his encouragement throughout the course of this study. I also wish to acknowledge the advice and assistance given by Dr Tony Cashmore of the Applied Biochemistry Division, DSIR, Palmerston North. Thanks are also extended to the Soil Science and Dairy Husbandry Departments, Massey University for the use of scintillation and gamma spectrometers.

LIST OF ABBREVIATIONS

AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BPB	Bromophenol blue
CM-	Carboxymethyl-
DE-	Diethylaminoethyl-
DNA	Deoxyribonucleic acid
cDNA	Copy DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
FBP	D-Fructose 1,6-bisphosphate
FBPase	Fructose-1,6-bisphosphatase
F6P	D-Fructose 6-phosphate
GTP	Guanosine 5'-triphosphate
Hab	Immunoglobulin heavy chain
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HK(x)M	50 mM Hepes, x mM KCl, 5 mM MgCl <sub>2</sub> , pH 7.4
HNa(x)M	50 mM Hepes, x mM NaCl, 5 mM MgCl <sub>2</sub> , pH 7.4
Lab	Immunoglobulin light chain
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate
PB	Phosphate buffer (10 mM Na <sub>2</sub> HPO <sub>4</sub> , 15 mM NaCl, pH 7.2)
PBS	Phosphate buffered saline (10 mM Na <sub>2</sub> HPO <sub>4</sub> , 150 mM NaCl, pH 7.4)
PEG	Polyethylene glycol
PFK	Phosphofructokinase
PMSF	Phenylmethyl sulphonyl fluoride
poly(A) <sup>+</sup>	RNA having a polyadenylate tract at its 3' terminus
poly(A) <sup>-</sup>	RNA lacking a polyadenylate tract at its 3' terminus
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N'',N''-Tetramethylethylenediamine
TMV	Tobacco Mosaic Virus
Tris	tris(Hydroxymethyl)aminomethane

## LIST OF FIGURES

FIGURE	PAGE
3.1 Elution of fructose-1,6-bisphosphatase from phosphocellulose with fructose 1,6-bisphosphate	31
3.2 Non-denaturing gel (7%) electrophoresis of fructose-1,6-bisphosphatase	32
3.3 SDS gel (10%) electrophoresis of fructose-1,6-bisphosphatase	33
3.4 Estimation of fructose-1,6-bisphosphatase subunit molecular weight by SDS gel (10%) electrophoresis	34
3.5 Effect of pH on fructose-1,6-bisphosphatase activity	35
4.1 SDS gel analysis of purified ovalbumin, rat albumin and rabbit gamma globulin	38
5.1 Ouchterlony immunodiffusion analysis of antisera against ovalbumin, rat albumin, FBPase and rabbit gamma globulin	46
5.2 Purification of antibodies to fructose-1,6-bisphosphatase by affinity chromatography on Sepharose-bound antigen	47
5.3 Ouchterlony immunodiffusion analysis of affinity-purified antibodies	48
5.4 Immunoprecipitation of $^{125}\text{I}$ -labelled anti-fructose-1,6-bisphosphatase by crude gamma globulin	49
5.5 Immunoprecipitation of $^{125}\text{I}$ -labelled anti-fructose-1,6-bisphosphatase by affinity-purified antibody	50
5.6 SDS gel (10%) analysis of affinity-purified antibodies	51
6.1 SDS gel (10%) analysis of FBPase immunoprecipitated from liver post-microsomal supernatant after a 24 hour interperitoneal labelling with [ $^{35}\text{S}$ ]methionine	58

FIGURE	PAGE
6.2 SDS gel (10%) analysis of albumin immunoprecipitated from serum after a 24 hour interperitoneal labelling with [ <sup>35</sup> S]methionine	59
6.3 SDS gel (10%) analysis of ovalbumin added to and immunoprecipitated from liver post-microsomal supernatant after a 24 hour interperitoneal labelling with [ <sup>35</sup> S]methionine	60
6.4 Semilog plot of molecular weight versus relative migration for protein subunits in albumin, FBPase and ovalbumin immunoprecipitates on 10% SDS gels	61
6.5 Fractionation scheme for the isolation of subcellular fractions from rat liver	63
6.6 SDS gel (7%) analysis of TCA-precipitable protein in liver supernatant and microsome fractions after a 10 minute pulse label of [ <sup>35</sup> S]methionine via the portal vein	65
6.7 SDS gel (7%) analysis of FBPase immunoprecipitated from liver supernatant and microsome fractions after a 10 minute pulse label of [ <sup>35</sup> S]methionine via the portal vein	67
6.8 SDS gel (7%) analysis of albumin immunoprecipitated from liver supernatant and microsome fractions after a 10 minute pulse label of [ <sup>35</sup> S]methionine via the portal vein	68
7.1 Isolation scheme for free and bound polysomes from rat liver	74
7.2 Sedimentation profiles of free and bound polysomes on isokinetic sucrose gradients	75
8.1 Binding of <sup>125</sup> I-labelled antibodies to free polysomes	83
8.2 Binding of <sup>125</sup> I-labelled antibodies to bound polysomes	84



FIGURE	PAGE
8.3 Effect of RNase on the binding of $^{125}\text{I}$ -labelled antibodies to bound polysomes	85
8.4 Binding of different concentrations of $^{125}\text{I}$ -labelled anti-albumin and anti-fructose-1,6-bisphosphatase to free and bound polysomes	86
8.5 Relative amount of $^{125}\text{I}$ -labelled anti-fructose-1,6-bisphosphatase binding to free and bound polysomes	87
8.6 Relative amount of $^{125}\text{I}$ -labelled anti-albumin binding to free and bound polysomes	88
8.7 Binding of $^{125}\text{I}$ -labelled anti-albumin and anti-fructose-1,6-bisphosphatase to different concentrations of free and bound polysomes	89
9.1 Time course of protein synthesis on isolated free and bound polysomes incubated <u>in vitro</u>	96
9.2 SDS gel (10%) analysis of TCA-precipitable protein produced in free and bound polysome incubations	97
9.3 SDS gel (10%) analysis of fructose-1,6-bisphosphatase immunoprecipitated from free and bound polysome incubations	98
9.4 SDS gel (10%) analysis of albumin immunoprecipitated from free and bound polysome incubations	99
10.1 Radiochemical purity analysis of L- $^{35}\text{S}$ methionine by TLC on cellulose	115
10.2 Formamide gel analysis of TMV RNA	116
10.3 Time course of protein synthesis and effect of RNA concentration on the translation of TMV RNA in wheat germ extracts	117

FIGURE	PAGE
10.4 Effect of $K^+$ and $Mg^{2+}$ concentration on the translation of TMV RNA in wheat germ extracts	118
10.5 SDS gel (7%) analysis of translation products produced in wheat germ extracts programmed with TMV RNA	119
10.6 Isolation of $poly(A)^+$ RNA by chromatography of rat liver polysomal RNA on $poly(U)$ -Sephadex	120
10.7 Non-denaturing gel (2.4%) analysis of $poly(A)^+$ and $poly(A)^-$ RNA from free polysomes	121
10.8 Non-denaturing gel (2.4%) analysis of $poly(A)^+$ and $poly(A)^-$ RNA from bound polysomes	122
10.9 Time course of protein synthesis and effect of spermine concentration on the translation of $poly(A)^+$ RNA in wheat germ extracts	123
10.10 The effect of $K^+$ and $Mg^{2+}$ concentrations on the translation of $poly(A)^+$ RNA from total polysomes in wheat germ extracts	124
10.11 Effect of RNA concentration on the translation of $poly(A)^+$ and $poly(A)^-$ RNA from free and bound polysomes in wheat germ extracts	125
10.12 SDS gel (7%) analysis of released products produced in wheat germ extracts programmed with $poly(A)^+$ RNA from free and bound polysomes	126
10.13 SDS gel (7%) analysis of ribosome-bound translation products produced in wheat germ extracts programmed with $poly(A)^+$ RNA from free and bound polysomes	127
10.14 SDS gel (10%) analysis of translation products produced in wheat germ extracts programmed with $poly(A)^+$ RNA from free and bound polysomes which were immunoprecipitated with anti-fructose-1,6-bisphosphatase	129

- 10.15 SDS gel (10%) analysis of translation products produced in wheat germ extracts programmed with poly(A)<sup>+</sup> RNA from free and bound polysomes which were immunoprecipitated with anti-albumin

## LIST OF TABLES

TABLE	PAGE
3.1 Purification of fructose-1,6-bisphosphatase from rat liver	30
5.1 Antibody purification by affinity chromatography	45
5.2 Antibody quantitation	48
6.1 Immunoprecipitation of albumin from serum, FB Pase and carrier ovalbumin from liver post-microsomal supernatant after a 24 hour interperitoneal labelling with [ <sup>35</sup> S]methionine	62
6.2 Distribution of TCA-precipitable protein in liver subcellular fractions after a 10 minute pulse label of [ <sup>35</sup> S]methionine via the portal vein	64
6.3 Distribution of radioactivity in albumin and FB Pase immunoprecipitates from liver supernatant and microsome fractions after a 10 minute pulse label of [ <sup>35</sup> S]methionine via the portal vein	66
7.1 Sedimentation distribution of polysome size classes	76
9.1 Distribution of immunoprecipitable albumin and fructose-1,6-bisphosphatase peptides synthesized by isolated free and bound polysomes <u>in vitro</u>	100
10.1 Distribution of immunoprecipitable albumin and fructose-1,6-bisphosphatase peptides produced in wheat germ extracts programmed with poly(A) <sup>+</sup> RNA from free and bound polysomes	128

## TABLE OF CONTENTS

	PAGE
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
LIST OF ABBREVIATIONS	v
LIST OF FIGURES	vi
LIST OF TABLES	xi
CHAPTER 1 INTRODUCTION AND HISTORICAL REVIEW	
1.1 The intracellular sites of protein synthesis in animal cells	1
1.2 The segregation of secretory proteins during protein synthesis	5
1.3 Objectives of this investigation	10
1.4 The discovery and isolation of fructose-1,6-bisphosphatase	11
1.5 Physical and enzymic properties of fructose-1,6-bisphosphatase	12
1.6 Metabolic regulation by fructose-1,6-bisphosphatase <u>in vivo</u>	14
CHAPTER 2 GENERAL METHODS	
2.1 Introduction	18
2.2 Experimental animals	18
2.3 Centrifugation	18
2.4 Ultrafiltration	18
2.5 Spectrophotometry	19
2.6 Protein and RNA determination	19
2.7 Polyacrylamide gel electrophoresis of proteins	
2.7.1 Discontinuous non-denaturing gels	19
2.7.2 Sodium dodecyl sulphate continuous denaturing gels	20
2.8 Estimation of protein subunit molecular weights	21
2.9 Polyacrylamide gel electrophoresis of RNA	
2.9.1 Continuous non-denaturing gels	22
2.9.2 Formamide denaturing gels	23
2.10 Determination of radioactivity	
2.10.1 Gel slices	24
2.10.2 Trichloroacetic acid-precipitable protein	24

	PAGE	
CHAPTER 3	PURIFICATION OF FRUCTOSE-1,6-BISPHOSPHATASE FROM RAT LIVER	
3.1	Introduction	25
3.2	Materials	25
3.3	Methods	
3.3.1	Assay of fructose-1,6-bisphosphatase	26
3.3.2	Purification of fructose-1,6-bisphosphatase	26
3.3.3	Treatment of animals with triamcinolone and alloxan	27
3.3.4	Quantitation of purified enzyme protein	28
3.4	Results and Discussion	28
CHAPTER 4	PURIFICATION OF OVALBUMIN, RAT ALBUMIN AND SHEEP GAMMA GLOBULIN	
4.1	Introduction	36
4.2	Materials	36
4.3	Methods and Results	
4.3.1	Ovalbumin	36
4.3.2	Rat albumin	36
4.3.3	Rabbit gamma globulin	37
CHAPTER 5	ANTIBODY PRODUCTION AND PURIFICATION	
5.1	Introduction	39
5.2	Materials	39
5.3	Methods	
5.3.1	Immunization protocols	39
5.3.2	Separation of gamma globulin from serum	40
5.3.3	Antibody specificity	40
5.3.4	Preparation of Sepharose-bound antigen affinity columns	41
5.3.5	Affinity chromatography	41
5.3.6	Antibody titre	42
5.4	Results and Discussion	42
CHAPTER 6	LABELLING OF LIVER PROTEINS <u>IN VIVO</u>	
6.1	Introduction	52
6.2	Materials	53
6.3	Methods	

	PAGE
6.3.1 Interperitoneal (24 hour) labelling	53
6.3.2 Intraportal (10 minute) labelling	54
6.3.3 Analysis of labelled products	54
6.3.4 Immunoprecipitation assay	55
6.4 Results and Discussion	55
 CHAPTER 7 POLYSOME ISOLATION	
7.1 Introduction	69
7.2 Materials	70
7.3 Methods	
7.3.1 Preparation of free and bound polysomes	70
7.3.2 Preparation of total polysomes	72
7.3.3 Polysome sedimentation analysis	72
7.4 Results and Discussion	72
 CHAPTER 8 <sup>125</sup> I-LABELLED ANTIBODY BINDING TO POLYSOMES	
8.1 Introduction	77
8.2 Materials	78
8.3 Methods	
8.3.1 Polysome preparation	79
8.3.2 Antibody iodination	79
8.3.3 Polysome binding experiments	79
8.4 Results and Discussion	80
 CHAPTER 9 PROTEIN SYNTHESIS BY ISOLATED POLYSOMES <u>IN VITRO</u>	
9.1 Introduction	90
9.2 Materials	91
9.3 Methods	
9.3.1 Cell sap preparation	91
9.3.2 Translation assays	92
9.3.3 Analysis of translation products	92
9.3.4 Immunoprecipitation assay	92
9.4 Results and Discussion	93
 CHAPTER 10 PROTEIN SYNTHESIS IN WHEAT GERM EXTRACTS <u>IN VITRO</u>	
10.1 Introduction	101
10.2 Materials	102
10.3 Methods	

	PAGE
10.3.1 Radiochemical purity of L-[ <sup>35</sup> S]methionine	103
10.3.2 Preparation of Tobacco Mosaic Virus RNA	103
10.3.3 Extraction of poly(A) <sup>+</sup> RNA from polysomes	104
10.3.4 Preparation of wheat germ extracts	106
10.3.5 Translation assays	106
10.3.6 Analysis of translation products	107
10.3.7 Immunoprecipitation assay	107
10.4 Results and Discussion	108
CHAPTER 11 DISCUSSION	131
BIBLIOGRAPHY	136