

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.

**EXPLORATIONS INTO THE NATURE OF
INSULIN BINDING TO OXIDIZED DEXTRAN**

This thesis was presented in partial fulfillment of the requirements for the
degree of Master of Science in Chemistry at Massey University

Yuming Li

1998

Abstract

The results reported in this thesis comprise an investigation into the conjugation of insulin to oxidized dextran, various release studies from the conjugates, and an attempt to interpret the binding nature of the conjugates. A model system involving the sustained release from insulin-dextran conjugates has been employed in this study. For insulin, up to 3 potential sites only (A1-Gly, B1-Phe and B29-Lys) were expected to bind to oxidized dextran. The rate of release and the maintenance of activity of the released protein are vital to such systems. Success in the interpretation of the binding nature of the conjugate will allow us to investigate its relationship to the rate of release. The desired rate of release for the sustained release of protein could then be achieved, once the projected binding could be obtained.

Activation of dextran was achieved by periodate oxidation to give levels of 8%, 16% and 27% oxidized dextran. Insulin was chosen for its relatively 'uncomplicated' structure and few possible sites available for binding with activated dextran. Insulin was bound to the dextran through imine bonds. Complex formation was examined under a wide range of conditions. Initial studies were begun with the determination of a desirable basic molar ratio. A molar ratio of insulin to 8% activated dextran of 10 : 1 arose from this set of experiments. Insulin was bound to 27% activated dextran at pH 7.4, pH 9 and pH 10. In the cases of pH 9 and pH 10, many more lower MW complexes were formed than at pH 7.4. It seemed that the higher the pH of formation, the more crosslinks occurred between an insulin molecule and dextran molecules in the lower MW range. Approximate physiological pHs (pH 7.1-7.8) were used for complex formation in all subsequent experiments.

Release studies were carried out under approximate physiological conditions (pH 7.4, 37°C). Immediate release was observed upon isolation by size exclusion chromatography. The greatest release occurred in the first 24 hours for all three activation levels. The higher the activation level of dextran, the lower the level of release. An equilibrium was established after several days' release and studies at 37°C produced the expected result: greater release relative to ambient.

A number of studies were carried out with complex after sodium cyanoborohydride had been used to reduce the imine bonds.

The first set of experiments on the reduced complexes was enzymatic cleavage studies, which employed trypsin and α -chymotrypsin. The results for trypsin digestion of the reduced insulin-27% oxidized dextran complex indicated partial binding had occurred at B29-Lys, in combination with full binding at B1 and/or A1. Amino acid analysis results of the isolated complex after trypsin digestion indicated about 90% binding occurred at B29-Lys for the complex, which formed at pH 7.1. The results of α -chymotrypsin digestion study were shown questionable due to its incomplete cleavage.

The reduced complexes were analyzed by amino acid analysis. The insulin-27% activated dextran complexes formed at pH 7.4, pH 9 and pH 10 showed similar extents of binding at B1-Phe, indicating B1 might be the prime binding site. There was more binding at B29 and A1 for the pH 9 than at pH 7.4 case. At pH 10 abnormal values arose. The studies for the complexes of insulin with 16% and 27% activated dextran indicated the more highly activated the dextran, the greater the binding at B29 and A1.

Trials with the 2, 4-dinitrophenyl-derivatization method proved to be a useful way to examine the degree of B1 and B29 binding from the amino acid analysis results of complex. The insulin-16% activated dextran complex formed at pH 7.1 was found to be about 100% binding at B1, 60% at A1 and 50% at B29.

Oxidative and reductive cleavage studies of A and B chains of insulin and the complex were carried out to investigate the level of A1 binding. After chemical cleavage of the three disulfide bonds in insulin and subsequent chromatography, the amino acid analysis results for the treated complexes indicated a significant proportion of A chain had bound to dextran, i.e. at A1. An estimation of 60-70% of A1 binding was achieved for this study.

This exploratory study has shown that varied complex formation conditions such as the level of activation of dextran, pH, and temperature could alter the extent of binding between insulin and dextran molecules. Amino acid analysis of the reduced complex was a useful method to interpret the binding.

ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisor Associate Professor David R.K. Harding for his guidance and assistance in helping me complete my Masterate and the opportunity to be a member of the Separation Science Group.

I wish also to thank Dick Poll for his constant instruction and assistance in helping me to become familiar with the use of the equipment (HPLC, FPLC and SMART system). A special thanks to Louisa Fisher, who as a fellow research student, has helped me a great deal at the beginning of my research and with the final graph treatment. And thanks to the other members of the separation of the separation science group, Simon Burton, Jennifer Cross, Elana Gorrie, Helen Guo and Rekha Parshot.

I wish also to thank Dr. Gill Norris and Jo Mudford for running my mass spectral analysis samples, and special acknowledgement to Debbie Frumau for her best efforts in running my amino acid analysis samples even though the problems occurred with the AA analyzer.

Furthermore, thanks to all the academic staff and other members of Chemistry Section, Institute of Fundamental Sciences, especially Professor Andrew M. Brodie and Associate Professor Paul D. Buckley for their support and suggestion through my two years study at Massey.

~~I would like to thank members of Associate Professor Len F. Blackwell's lab, especially ZhanDong Zhao and Yinqiu Wu.~~

Finally I would like to thank my wife, my parents and my brother for all their constant support.

TABLE OF CONTENTS

Abstract	ii
Acknowledgements	iv
Table of Contents	v
List of Figures	viii
List of Tables and Schemes	xi
List of Abbreviation	xiii
CHAPTER ONE INTRODUCTION	1
1.1 Drug Delivery	1
1.2 Controlled Release Systems	2
1.3 Polymer-Drug Conjugates	4
1.4 Modification of Proteins	6
1.5 Periodate Oxidation of Dextran	8
1.6 Imine Formation	11
1.7 Complex Formation of Protein with Dextran	12
1.8 Sustained Release of Proteins from Dextran	15
1.9 Investigations into the Binding Sites of Insulin-Activated Dextran and Subsequent Release	17
CHAPTER TWO MATERIALS AND METHODS	18
2.1 Reagents and Equipment	18
2.2 Periodate Oxidation	19
2.3 Iodometric Titration	19
2.4 Complex Formation	19
2.5 Complex Release	20
2.6 Complex Reduction Studies	20
2.7 Trypsin Digest and Peptide Mapping	20
2.8 Chymotrypsin Digest and Peptide Mapping	21
2.9 Preparation of DNP-Insulin	22
2.10 Oxidative Cleavage of Disulfide Bonds of Insulin	22
2.11 Reductive Cleavage of Disulfide Bonds of Insulin	24

2.12	Mass Spectrum Sample Preparation	25
2.13	Amino Acid Analysis Preparation	25

CHAPTER THREE COMPLEX FORMATION AND RELEASE STUDIES **26**

3.1	Introduction	26
3.2	Results and Discussion	29
3.2.1	Periodate Oxidation of Dextran	29
3.2.2	Initial Studies of Insulin	31
3.2.3	Complex Formation	33
3.2.3.1	Determination of Initial Molar Ratio	33
3.2.3.2	Differences in Various pHs	37
3.2.3.3	Other Conditions for Complex Formation Studies	40
3.2.4	Complex Release Studies	41
3.2.5	Complex Reduction Studies	49

CHAPTER FOUR ENZYMATIC CLEAVAGE STUDIES WITH TRYPSIN AND α -CHYMOTRYPSIN **55**

4.1	Introduction	55
4.2	Results and Discussion	57

CHAPTER FIVE ANALYSIS OF INSULIN BINDING TO OXIDIZED DEXTRAN BY AMINO ACID ANALYSIS **65**

5.1	Introduction	65
5.2	Results and Discussion	68
5.2.1	Initial Complex Binding Studies by AAA	68
5.2.2	AAA Studies for Complexes Formed at Different pHs	70
5.2.3	AAA Studies for Different Extent of Activation of Dextran at pH 7.4	75
5.2.4	AAA Studies for DNP-Insulin Derivatives	76

5.2.5	Final AAA Study for Insulin-Dextran Complex	78
CHAPTER SIX	CHEMICAL CLEAVAGE STUDIES OF THE A AND B CHAINS OF INSULIN	81
6.1	Introduction	81
6.2	Results and Discussion	83
CHAPTER SEVEN	CONCLUSIONS AND FUTURE WORK	95
7.1	Conclusions	95
7.2	Future Work	99
REFERENCE		101

LIST OF FIGURES

Figure 1.5.1	Periodate oxidation of dextran	9
Figure 1.5.2	Overall reaction individual glucose molecule periodate oxidation	10
Figure 1.5.3	Relationship between degree of oxidation and % double oxidized residues	11
Figure 1.7.1	Extent of complex formation over increasing dextran activation levels for a 24hr period	13
Figure 1.7.2	Possible structure of protein-dextran complex	14
Figure 3.1.1	The primary structure and 3-D structure of monomeric porcine insulin	27
Figure 3.2.1.1	Periodate oxidation of dextran	31
Figure 3.2.2.1	Absorbance wavelength scan of porcine insulin	32
Figure 3.2.2.2	Reverse-phase analytical chromatogram of porcine insulin	32
Figure 3.2.2.3	Analytical capillary Electrophoretogram of porcine insulin	33
Figure 3.2.3.1.1	Complex ($\uparrow\uparrow$) formation over time for insulin (\uparrow) and 8% activated dextran with a molar ration of 5 : 1	34
Figure 3.2.3.1.2	Complex ($\uparrow\uparrow$) formation over time for insulin (\uparrow) and 8% activated dextran with a molar ration of 10 : 1	35
Figure 3.2.3.1.3	Complex ($\uparrow\uparrow$) formation over time for insulin (\uparrow) and 8% activated dextran with a molar ration of 15 : 1	36
Figure 3.2.3.2.1	Complex ($\uparrow\uparrow$) formation for insulin (\uparrow) and 27% activated dextran at (a) pH 7.4, (b) pH 9 and (c) pH 10 at 48 hrs.	38
Figure 3.2.3.2.2	Molecular weight distribution by SEC of Dextran T-40	39
Figure 3.2.3.3.1	Complex ($\uparrow\uparrow$) formation over time for insulin (\uparrow) and 8% activated dextran (a) without shaking and (b) with shaking	41
Figure 3.2.4.1	Release of insulin-like species (\downarrow) from complex ($\downarrow\downarrow$)	42
Figure 3.2.4.2	Release of insulin-like species from complex (insulin- 8% activated dextran) over time	43
Figure 3.2.4.3	Release of insulin-like species from complex (insulin- 16% activated dextran) over time	44

Figure 3.2.4.4	Release of insulin-like species from complex (insulin-27% activated dextran) over time	45
Figure 3.2.4.5	Reverse phase chromatography of the isolated released insulin-like species for mass spectrum after SCE isolation	47
Figure 3.2.4.6	Reverse phase chromatography of the released insulin-like species from release mixture	47
Figure 3.2.4.7	Mass spectrum for release insulin	48
Figure 3.2.5.1	SEC chromatograms of reduced complex and control experiment	50
Figure 3.2.5.2	Comparison of release from (a) non-reduced complex and (b) reduced complex	51
Figure 3.2.5.3	Insulin (\downarrow) release study from reduced complex (\Downarrow) by SMART system (Superdex 75)	53
Figure 3.2.5.4	Insulin monomer, dimer: three-dimensional atomic structure	54
Figure 4.2.1	Peptide mapping of trypsin digestion of insulin and reduced complex	57
Figure 4.2.2	Mass spectrum for the small fragment of insulin trypsin digestion	58
Figure 4.2.3	Mass spectrum for the large fragment of insulin trypsin digestion	59
Figure 4.2.4	Mass spectrum for heptapeptide-like peak from reduced complex trypsin digestion	60
Figure 4.2.5	Peptide mapping of α -chymotrypsin digestion of insulin and reduced complex on FPLC	63
Figure 4.2.6	Peptide mapping of α -chymotrypsin digestion of insulin and reduced complex on SMART system	64
Figure 5.1.1	Automatically recorded chromatographic analysis of amino acids on a cation-exchange resin	65
Figure 6.2.1	Analytical capillary electrophoretograms of (a) insulin and (b) oxidized insulin by performic acid	83
Figure 6.2.2	Analytical capillary electrophoretograms of (a) oxidized B chain and (b) oxidized insulin-dextran complex	85

Figure 6.2.3	SEC chromatogram of reduced complex after performic acid treatment	85
Figure 6.2.4	Peptide mapping of oxidative cleavage of insulin and reduced complex	87
Figure 6.2.5	Peptide mapping of reductive cleavage of insulin and reduced complex	89
Figure 6.2.6	Mass spectrum fragment A from reductive cleavage of insulin	90
Figure 6.2.7	Mass spectrum fragment B from reductive cleavage of insulin	91
Figure 6.2.8	Mass spectrum fragment A from reductive cleavage of reductive insulin-dextran complex	92
Figure 6.2.9	Mass spectrum fragment B from reductive cleavage of reductive insulin-dextran complex	92

LIST OF TABLES AND SCHEMES

Scheme 1.3.1	Drug release mechanism	4
Table 3.2.1.1	Typical results for activation of dextran	30
Table 3.2.4.1	Release study of insulin-8% activated dextran complex by SMART system (Superdex 75)	43
Table 3.2.4.2	Release study of insulin-16% activated dextran complex by SMART system (Superdex 75)	45
Table 3.2.4.3	Release study of insulin-27% activated dextran complex by SMART system (Superdex 75)	45
Table 3.2.4.4	Release study of insulin-16% activated dextran complex by SMART system (Superdex 75) at ambient	46
Scheme 3.2.5.1	Cyanoborohydride reduction of insulin-dextran complex	50
Scheme 4.1.1	The cleavage sites of insulin by trypsin (\uparrow) and α -chymotrypsin (\uparrow)	56
Table 4.2.1	AAA for the complex after trypsin treatment	61
Scheme 5.1.1	Acid hydrolysis of reduced insulin-dextran complex	66
Scheme 5.1.2	Formation of DNP-amino acid	67
Table 5.2.1	Amino acid composition with respect to arginine of the complex in comparison to insulin and literature sequence	69
Table 5.2.2.1	AAA for complex of 27% activated dextran and porcine insulin formed at pH 7.4, pH 9 and pH 10	71
Scheme 5.2.2.1	Acidic and basic nature of an amino acid	72
Scheme 5.2.2.2	Mechanism of imine formation by reduction of an aldehyde with a primary amine	73
Table 5.2.2.2	Amino acid composition with respect to arginine of the complex formed at pH 7.4 and pH 9 in comparison to insulin literature sequence	74
Table 5.2.3.1	Amino acid composition with respect to arginine of the complexes (insulin-16% activated dextran and insulin-27% activated dextran) in comparison to insulin literature sequence	76

Table 5.2.4.1	Amino acid composition with respect to arginine of the DNP-insulin-dextran complex in comparison to the complex and literature sequence	77
Table 5.2.5.1	Amino acid composition with respect to arginine of insulin-16% activated dextran complex in comparison to insulin literature sequence	79
Table 6.2.1	Amino acid composition with respect to arginine of the complex after oxidative cleavage in comparison to insulin A chain and B chain sequences	86
Table 6.2.2	Amino acid composition with respect to arginine of the complex after reductive cleavage in comparison to insulin A chain and B chain sequences	93
Table 7.1.1	Binding extent of three sites	99

LIST OF ABBREVIATIONS

A1-Gly	α -NH ₂ terminal group glycine of A chain of insulin
AAA	amino acid analysis
Ab	antibody
B1-Phe	α -NH ₂ terminal group phenylalanine of B chain of insulin
B29-Lys	ϵ -lysine of the 29 th residue from the N-termini of B chain of insulin
CD4	cell surface glycoprotein receptor for HIV
CE	capillary electrophoresis
DOR	double oxidized residues
ELISA	enzyme-linked immunosorbent assay
EPC	especially for small proteins
FDA	food and drug administration
FPLC	fast protein liquid chromatography
GP120	glycoprotein-120
HPLC	high performance liquid chromatography
Met-hGH	recombinant methionyl human growth hormone
MW	molecular weight
MWCO	molecular weight cut off
NaBH ₄	sodium borohydride
NaBH ₃ CN	sodium cyanoborohydride
PBu ₃	tributylphosphine
PEG	polyethylene glycol
PHPMA	N-2-hydropropyl methacrylamide
rhGH	recombinant human growth hormone
rIGF-1	recombinant human insulin-like growth factor-1
RIA	radioimmunoassay
RPLC	reverse phase liquid chromatography
rtPA	recombinant human tissue plasminogen activator
SEC	size exclusion chromatography
TFA	trifluoroacetic acid
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
Tris	tris-(hydroxymethyl)-aminomethane

Abbreviations used for amino acids:

Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Cysteine	Cys
Glutamic acid	Glu
Glutamine	Gln
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tyrosine	Tyr
Tryptophan	Trp
Valine	Val
Asx	asparagine and aspartic acid
Glx	glutamine and glutamic acid