EXPLORATIONS INTO THE NATURE OF INSULIN BINDING TO OXIDIZED DEXTRAN

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Yuming Li

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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-derivativatization 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.

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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
$NaBH_3CN$	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 asparatic acid
Glx	glutamine and glutamic acid