MECHANISTIC AND FUNCTIONAL STUDIES OF ZINC (II) ACTIVATION OF C-PEPTIDE AND ITS EFFECT ON RED BLOOD CELL METABOLISM

By

Wathsala Medawala

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ABSTRACT

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C-peptide, a 31 amino acid peptide co-secreted with insulin from β-cells in the pancreas, has long been considered a by-product of insulin synthesis. Later it was discovered that C-peptide could ameliorate diabetic complications such as neuropathy, nephropathy, retinopathy and microvascular disease. The molecular mechanism behind the effects of C-peptide is not yet completely understood. C-peptide improves the microcirculation by increasing endothelial-derived nitric oxide (NO), red blood cell (RBC) deformability and Na^{+}/K^{+}-ATPase activity. C-peptide increases glucose utilization and ATP release from RBCs and endothelial cells. ATP is a stimulus for the production of endothelial-derived NO, which is a known vasodilator. Therefore, the C-peptide mediated increase in RBC-derived ATP release can lead to improved blood flow. C-peptide alone did not increase RBC-derived ATP release. The presence of a metal ion such as Zn^{2+} was needed to elicit this response. However, the role of Zn^{2+} in C-peptide activity is not fully understood. A fluorescence based study was used to investigate the binding of Zn^{2+} to C-peptide. Results indicate that C-peptide binds one Zn^{2+} ion and with a binding constant of $1.02 \times 10^{7}\ M^{-1}$ at pH 5.5 (pH inside a mature β-cell granule). At physiological pH (pH 7.4), C-peptide binds with two Zn^{2+} ions with a binding constant of $7.99 \times 10^{6}\ M^{-1}$. This suggests that C-peptide may bind with Zn^{2+} inside the β-cell granule and release Zn^{2+} upon entering the blood stream. Circular dichroism studies suggest that a 1:1 Zn^{2+} to C-peptide ratio elicits a decrease in

the randomness of the peptide chain. Studies using five single amino acid peptide mutants of C-peptide show that substitution of glutamate residues at positions 27, 11 and 3 with alanine decreased the Zn^{2+} binding to C-peptide by ~50%, agreeing with the activity of these mutants in other bio-assays. Several studies have indicated the necessity of insulin for C-peptide activity. The Zn^{2+} that is available with insulin hexamers may be responsible for the activation of C-peptide. Using a RBC-derived ATP assay, it was shown that Zn^{2+} added in the form of Zn-insulin activates C-peptide, as observed by the increase in ATP release. The C-peptide interaction with RBCs was studied using an ELISA. Zn^{2+}-activated C-peptide bound to RBCs in a dose dependent manner, 2 pmol of Zn^{2+}-activated C-peptide binding to 1 mL of 7% red blood cell sample at saturation (~1500 molecules/ cell). The interaction did not change significantly even in the absence of Zn^{2+}. However, Zn^{2+} uptake in RBCs was observed only in the presence of C-peptide, indicating a possible role for C-peptide as a Zn^{2+} carrier.

One argument against C-peptide being a potential medication is that patients with type 2 diabetes develop the complications of the disease despite having circulating C-peptide. Red blood cells incubated in high glucose (>10 mM), showed lower interaction with Zn^{2+}-activated C-peptide and lower ATP release, showing that RBCs of patients with uncontrolled blood glucose levels might show resistance to the action of the peptide. Also, the exposure of Zn^{2+}-activated C-peptide to serum albumin and mM levels of other cations and anions decreases its activity. Taken together, the work presented in this dissertation explains the role of Zn^{2+} in C-peptide action and how reproducible results may be obtained in C-peptide clinical trials.

To Kaveesh and Lasantha

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TABLE OF CONTENTS

LIST OF TABLES

.

LIST OF FIGURES

Chapter 1 - Introduction

1.1 Diabetes Mellitus

Diabetes is a group of metabolic disorders with the common characteristic feature of hyperglycemia as a result of defects in insulin production, insulin action or both.[1] According to 2011 National Diabetes Fact Sheet, 25.8 million Americans, or 8.3% of the population, are affected by diabetes. The disease is more widespread among the elderly population and in non-Hispanic blacks, Hispanics, Native Americans and Asians.[2] In the United States (US), although deaths due to cancer, stroke and cardiovascular disease are decreasing, the death rate due to diabetes has increased by more than 30% in the last two decades and life expectancy of diabetic patients is 15 years less than that of healthy individuals.[3] Therefore, it is apparent that diabetes has become a major cause of mortality and morbidity in the US.

The impact of the disease is not limited to the US. Urbanization, increasing levels of obesity, and decreasing levels of physical activity have led the way to an increase in diabetes in the world population, with the number of people with diabetes worldwide having reached 171 million by the year 2000.[4] It is estimated that from 1995 to 2025 there will be a 122% increase in the number of people having diabetes, and the prevalence in underdeveloped countries will increase at a faster rate than in developed countries.[5]

The economic impact of diabetes is vast. According to the American Diabetes Association, the direct economic cost of the disease was $116 billion for the US alone in 2007. When indirect costs due to loss of productivity, early mortality and disabilities were taken into

consideration, this number approached $174 billion.[6] Treatment of the disease has progressed from treatment of hyperglycemia with exogenous insulin to medications to improve insulin secretion, actions to control the onset of the disease by diet and exercising, and islet cell transplants.

1.2 Classification, Mechanism, Treatment and Complications of Diabetes Mellitus

Diabetes is a heterogeneous group of diseases that result in hyperglycemia via different mechanisms, and because the pathophysiological mechanisms are different, the treatments for diabetes differ as well. A patient is considered diabetic if the fasting plasma glucose level equals or exceeds 126 mg/dL (7 mM) or if the plasma glucose level is at or above 200 mg/dL (11.1 mM) two hours after a glucose tolerance test (75 g of glucose taken orally).[7] A fasting glucose level of 100-125 mg/dL is identified as impaired fasting glucose (IFG) and is considered to be a precursor to diabetes.[8] According to the National Health and Nutritional Evaluation Survey (NHANES), 26% of the US population over 20 years of age have IFG and are at a high risk of developing diabetes later in life.

Earlier classifications of diabetes considered the method of treatment, for example insulin dependent diabetes mellitus and non insulin dependent mellitus, rather than the pathophysiology. Currently, the ADA (American Diabetes Association) recommends classification that considers the mechanism causing hyperglycemia. According to ADA classification, there are four major categories: type 1 diabetes, type 2 diabetes, gestational diabetes and other specific forms of diabetes.

Type 1 diabetes, formerly known as juvenile diabetes or insulin dependent diabetes, is characterized by impaired insulin secretion. Insulin is the hormone necessary for glucose homeostasis in the body and regulates glucose uptake into muscle cells and adipose (fat) tissue cells, hence deficiency in the secretion of insulin leads to elevated blood sugar levels. The impaired insulin secretion in type 1 patients is due to an autoimmune destruction of the insulin secreting beta cells in the pancreas. The patient starts showing the symptoms of the disease when approximately 80% of the cells are destroyed. It is believed that the onset of type 1 diabetes is due to a combination of genetic susceptibility and environmental factors. Although not yet confirmed, the environmental factors are believed to be viral attacks such as rubella or enteroviruses, food additives such as nitrosamines, toxins or in some cases cow's milk.[9-11] The individual is believed to have an immune system prone to developing the disease, while one or more other factors trigger the immune attack on the beta cells. There is evidence of people with particular HLA (human leukocyte antigen) haplotypes developing the disease,[12] but again not all individuals with these haplotypes develop diabetes, suggesting that there is more than one factor controlling the onset of the disease.

Insulin resistance can be defined as the inability of a known quantity of insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population.[13] Type 2 diabetes, constituting about 90% of the diabetic population in the US, is a condition where the individual has insulin resistance in addition to relative insulin deficiency with increased gluconeogenesis in the liver.[14] It is believed that insulin resistance precedes insulin deficiency. Obesity and being overweight are two factors closely associated with insulin resistance, and it has been shown that weight loss helps slow down the progression from

impaired glucose tolerance (IGT) to type 2 diabetes.[15] However, insulin resistance itself does not cause diabetes. It is caused by subsequent failure of the beta cells to produce insulin. Before the disease develops, the beta cells compensate for insulin resistance by increasing the secretion of insulin. There is evidence for measurable beta cell hyperactivity in obese individuals who have insulin resistance.[16] The reason for failure of beta cells in type 2 patients is still not clear. However, studies have shown that prolonged exposure of islet cells to free fatty acids (FFA) suppresses insulin production.[17] It has been also observed that there are increased amounts of amyloid deposits in the islet cells of type 2 diabetic patients.[18] Amyloid deposits, made out of amylin, decrease the beta cell mass, and a correlation has been seen among the amount of dietary fat and formation of amyloids. Therefore, dietary fat seems to play a role in increasing hyperglycemia in type 2 patients. Similar to type 1 diabetes, type 2 diabetes is also considered to evolve due to genetic susceptibility and environmental factors such as obesity, decreased physical activity and age. Genetics is considered to play a larger role in causing type 2 diabetes than in type 1.[14]

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. GDM complicates about 4% of pregnancies within the US, or about 135,000 cases per year.[19] GDM complicates pregnancy by increasing the risk of preeclampsia (a pregnancy related blood-circulation problem that causes high blood pressure), fetal macrosomia (big baby syndrome) and caesarian delivery. Women who suffer from GDM have a higher risk of developing hypertension and diabetes after pregnancy. Increased insulin resistance is often considered as one of the major factors leading to GDM. Insulin resistance

increases during pregnancy, especially during the last trimester, due to increased maternal adipose tissue, placental hormones and increased insulin clearance by the placenta.[20] About 70% of women diagnosed with GDM develop type 2 diabetes within 10 years after delivery. Although not common, autoimmune attack on the islet cells may occur during pregnancy, which can lead to type 1 diabetes. These patients will rapidly progress towards diabetes after pregnancy and the disease can be diagnosed via an antibody screening.[14]

In addition to these three major types, there are other forms of diabetes. Any other disorder causing the destruction of pancreatic beta cells will eventually cause diabetes via decreasing insulin secretion. Some examples are cystic fibrosis, pancreatitis, pancreatic resection (surgical removal of the pancreas), hemochromatosis and trauma. The severity of the disease depends on the amount of beta cell mass lost. The loss of alpha cells along with beta cells in such an event can further disturb glucose homeostasis due to the decrease in glucagon secretion.[14]

Hormonal imbalances can also lead to diabetes. Some hormones, e.g. glucagon, growth hormone, epinephrine and cortisol, interfere with the action of insulin. Excess of these hormones can lead to increased insulin resistance and increased liver glucose production. Genetic defects in beta cell function can cause diabetes, collectively called maturity-onset diabetes of the young (MODY). Some genetic defects can alter insulin action via mutations in the insulin receptor. Leprechaunism (Donohue syndrome) and Rabson-Mendelhall syndrome are two such genetic syndromes. In all, the ADA has recognized more than 56 specific forms of diabetes.[1]

Diabetes is treated by insulin therapy, oral medications to increase insulin performance or overcome insulin resistance or a combination of these. Type 1 diabetic patients need external

insulin to maintain their blood glucose levels, especially after a meal. Cells of these patients are sensitive to insulin; therefore, insulin treatment alone can be used to keep the glucose levels under control. Type 2 diabetes is treated initially by a combination of diet, exercise and oral medication, such as metformin. Eventually, when the islet beta cells fail, type 2 patients will also require external insulin for survival. In the recent past, islet transplantation has been viewed as an ideal solution for diabetes and this area of research is still developing. According to the Collaborative Islet Transplant Registry annual report 2006, of the 225 patients who received islet transplant treatment, two thirds achieved 'insulin independence' during the year following transplantation. However, the patients eventually had to restart insulin treatment because the islet cell function started decreasing.[21]

Diabetes can cause both acute and chronic complications in patients. Most of the complications are the result of uncontrolled hyperglycemia. Acute complications include diabetic ketoacidosis (DKA), hyperosmolar hyperglycemic syndrome (HHS) and hypoglycemia. DKA is normally present in type 1 diabetic patients and caused by increased ketone bodies in blood. Decreased blood insulin levels cause hepatic ketone body production as a fuel for the brain and causes blood pH to drop, leading to acidemia and dehydration. Unresolved DKA can lead to coma and eventually death.[22] HHS is a condition with a higher degree of dehydration, hyperglycemia and lack of acidosis. Patients with HHS, commonly type 2 diabetic patients, have a greater insulin reserve. Compared to DKA, HHS has higher mortality rates.[23] The condition of hypoglycemia occurs when blood glucose levels decrease below 70 mg/dL. This occurs in diagnosed diabetic patients due to intensive glucose control. The fear of hypoglycemia is the major factor limiting diabetic patients from achieving euglycemia (healthy glucose level): an

insulin dose, higher than needed for the relevant glucose consumption, can send the patients into a state of hypoglycemia.[24] Symptoms include confusion or difficulty thinking, sleepiness, fatigue, hunger and, if left untreated, hypoglycemia can lead to seizures and coma. In addition, activation of the autonomic nervous system can cause sweating, palpitations, anxiety and paresthesias (the feeling of 'pins and needles').[25 p.119-128]

Vascular diseases, retinopathy (disease of the retina), neuropathy (disease or malfunction of the nerves) and nephropathy (kidney disease) are chronic complications of diabetes mellitus. Hyperglycemia induced vascular and neuronal changes that lead to diabetic complications include endothelial dysfunction, inflammatory cell activation and changes in vascular and neuronal factors.[26] There are several theories as to how hyperglycemia induces diabetic complications, including the aldose reductase (AR) theory, the advanced glycation endproduct (AGE) theory, the reactive oxygen intermediate (ROI) theory and the protein kinase C (PKC) theory.

In the AR theory, sometimes referred to as the polyol pathway theory, an increase in glucose concentration in the cell increases the flux of glucose through the enzyme aldose reductase, which reduces glucose to sorbitol using NADPH. The decline in NADPH level increases cellular oxidative stress, and the accumulation of sorbitol in the cell is believed to increase the osmotic vascular damage and contribute to development of cataracts.[27]

In AGE theory, diabetes is believed to be a form of accelerated aging, where proteins become irreversibly modified with sugars via the Maillard reaction to form AGEs.[28] Formation of AGEs impairs the function of the proteins, and causes damage to structural proteins like

collagen, as well as functional proteins like enzymes.[29, 30] In addition, AGEs can bind to cell receptors and trigger a cascade of cellular events that lead to abnormal cellular functions.[31]

Another mechanism that explains the adverse effect of hyperglycemia is the formation of ROIs. Glucose metabolism via mitochondrial oxidative phosphorylation can produce ROIs as byproducts. An increase in available glucose increases glucose metabolism, in turn producing more ROIs.[32] ROIs increase oxidative stress of the cell, damage cellular proteins, decrease NO levels and promote leukocyte (white blood cell) adhesion to the endothelium.[33-35]

Under normal conditions, the activation of protein PKC occurs via activation of phospholipase C and consequent increases in Ca^{2+} and diacylglycerol (DAG) levels. DAG and PKC control vascular functions such as endothelial activation, vasodilator release, growth factor signaling and permeability.[26] Under hyperglycemic conditions, elevated glycolysis produces increased levels of glycealdehyde-3-phosphate (G3P), which in turn can be converted to DAG. Elevated DAG levels cause the activation of PKC. In addition, PKC can also become activated indirectly by ROIs and AGEs. Pathological activation of PKC leads to vascular damage,[36] increased leukocyte adhesion[37] and alterations in blood flow.[38]

Among the chronic diabetic complications, vascular diseases, especially atherosclerosis, are the major cause of death and disability in diabetic patients. Vascular diseases can be attributed to abnormal endothelial and smooth muscle cell function and a high tendency for thrombosis. Endothelial dysfunction (mainly characterized by decreased nitric oxide (NO) production and expression of leukocyte adhesion molecules on the endothelium), decreased

bioavailability of NO and abnormal platelet function all lead to cellular events that cause atherosclerosis and increase the risk of cardiovascular disease.[39] In the circulatory system, NO has many important vasoprotective effects such as reducing platelet-vascular wall interactions, reducing the adhesion of monocytes to endothelial cells, and inhibition of vascular smooth muscle cell proliferation.[40] In diabetic patients, hyperglycemia and oxidative stress are strong inhibitors of NO-release from endothelial cells. Increased leukocyte-endothelial adhesion,[41] increased blood viscosity[42, 43] and changes in hemodyanamic properties of red blood cells[44, 45] in addition to endothelial dysfunction, lead to impaired tissue perfusion, and development of microvascular complications.[46]

Microvascular complications lead to other diabetic complications like retinopathy, neuropathy and nephropathy. Diabetic retinopathy (DR) accounts for 12% of new cases of blindness in the US each year.[47] In a population based study, within 15 years of diabetes diagnosis, 75% of the patients develop DR.[48] The changes in the retinal vascular function due to hyperglycemia, including retinal blood flow impairment, increased monocyte and leukocyte adhesion and capillary closure result in localized hypoxia.[49-51] One of the earliest changes in DR is the death of microvascular contractile cells (pericytes). Death of pericytes and the loss of intercellular contacts lead to endothelial cell proliferation and development of microaneurysms (swelling of the blood vessel).[52] When vascular damage interferes with blood flow to the retina it causes retinal ischemia (lack of blood and oxygen supply to the retina), which leads to an increased amount of vascular growth factors. These growth factors result in neovascular changes,

characterized by the growth of new blood vessels on the retina and posterior surface of the vitreous (Figure 1.1). These new blood vessels and contraction of accompanying fibrous tissue distort the retina and lead to retinal detachment. In addition, new blood vessels can bleed leading to vitreous hemorrhage. Both retinal detachment and non-clearing vitreous hemorrhage lead to loss of vision.[26] In addition, retinal vessel leaks result in thickening of the retina, termed as macular edema, which causes irreversible loss of central vision. For both type 1 and type 2 diabetes, the major risk factor for DR is the duration of diabetes. However, the prevalence of PDR is higher in type 1 diabetes than in type 2.[53]

In the US, diabetes mellitus is the most common cause of peripheral neuropathy, and almost all diabetic patients have some level of neuropathy (damage to the nervous system). Similar to retinopathy, incidence of diabetic neuropathy also increases with duration and severity of disease,[54] with type 1 patients showing a more rapid progression than type 2 patients.[55] Neuropathy can present itself as mononeuropathy (affecting one nerve or nerve group) and polyneuropathy (affecting multiple nerves).[56] Abnormalities in neurovascular flow and direct hyperglycemic damage to nerve tissue are thought to contribute to diabetic neuropathy.[57] The neurovascular complications resulting from a decrease in the endothelial derived vasodilator,

NO, or an increase in vasoconstrictors such as endothelin 1 (ET-1) bring about neuronal ischemia. Other microvascualar complications such as the death of pericytes, endothelial cell proliferation and monocyte adhesion to the endothelium have also been observed in neuropathy.[58, 59] Reduction in axonal Na^+/K^+ ATPase activity and decrease in neurotrophic factors lead to apoptosis of neuronal cells.[57, 60]

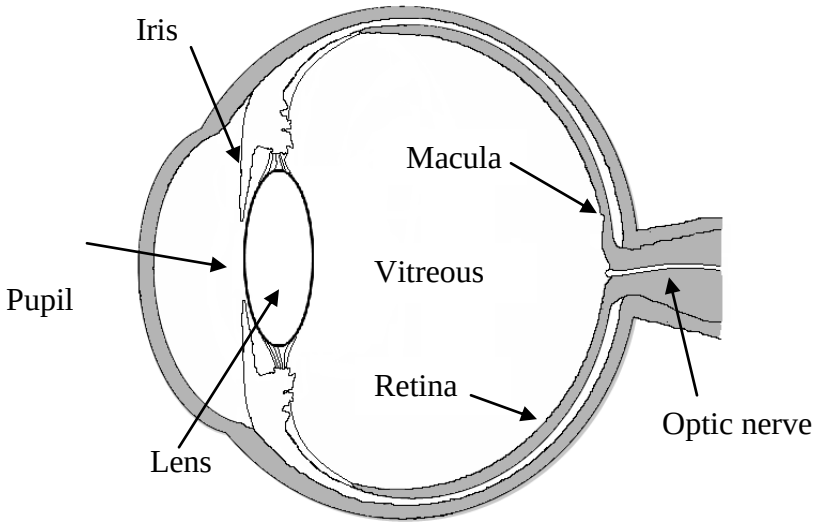

Figure 1.1: Schematic diagram of the cross-section of the human eye to show the anatomy.

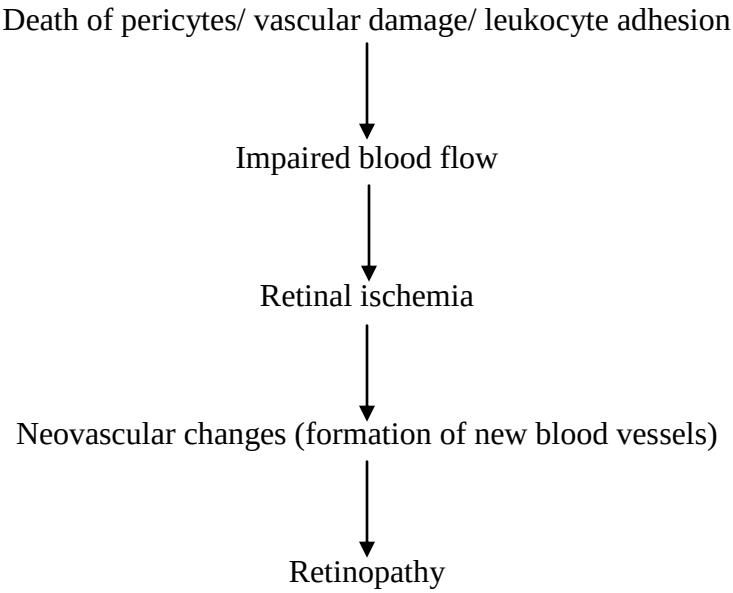

Figure 1.2: Flow diagram to show the changes in the retinal vascular system leading to retinopathy. Improvement of the blood flow leads to a decrease in the complications.

Peripheral neuropathy can lead to loss of sensation and when combined with microvascular and macrovascular complications is the major cause of nontraumatic amputations in the US.[61] Oxygen supplementation to neurons,[62] administration of vasodilatory agents,[63] and control of blood glucose[64] have all shown to have beneficiary effects on diabetic neuropathy under preclinical conditions and in animal models.

DM is the main cause of end stage renal disease in the US.[65] Renal disease occurs both in type 1 and type 2 diabetes, however type 2 diabetic patients are less likely to progress to overt renal failure.[25 p.156] Diabetic nephropathy is initially characterized by glomerular hyperfiltration and resulting microalbuminuria. Glomerular hyperfiltration can be attributed to the dilation of the afferent glomerular arteriole and the constriction of the efferent glomerular arteriole (Figure 1.3). This increases the hydrostatic pressure and forces the fluid through the filtration apparatus causing albuminuria, characterized by the presence of albumin in urine. This abnormality in vasodilation is caused by vasodilatory agents like NO and prostanoids.[66, 67] An increase in vascular endothelial growth factors (VEGF) also contributes to increased permeability in the filtration apparatus.[68] With the progress of the disease, changes in the synthesis of glomerular basement membrane macromolecules like collagen and proteoglycans increase the basement membrane thickness and lead to glomerular dysfunction.[69] This change in macromolecule synthesis is dependent on the action of several growth factors.[70, 71] In addition, the increase in collagen and decrease in proteoglycans in the extracellular matrix in the mesangial area cause the expansion of the mesangium (membrane supporting the capillary loops in the glomerulus), which

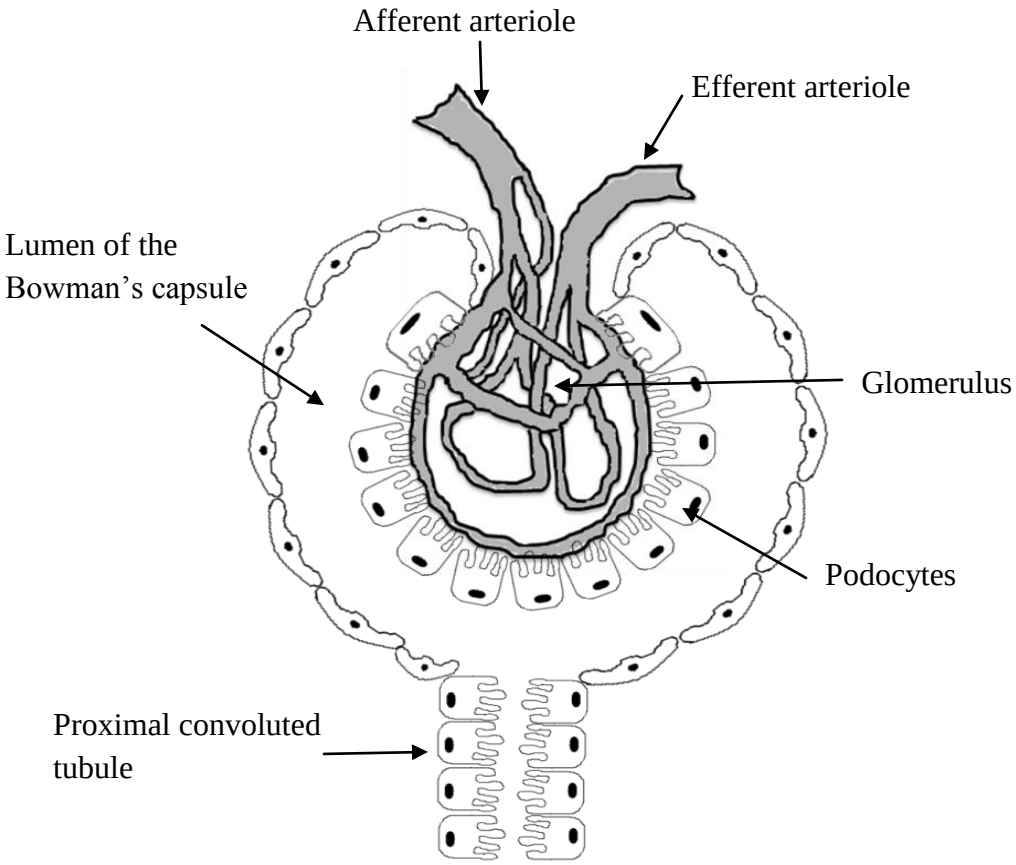

Figure 1.3: Schematic diagram of the filtration apparatus in the kidney showing afferent and efferent arterioles. Blood flow enters the glomerulus through the afferent arteriole. The glomerulus is semi-permeable and allows water and other soluble material to pass through and be excreted as urine. Glomerular hyperfiltration occurs due to dilation in the afferent arteriole and constriction in the efferent arteriole. C-peptide reverses this adverse effect by constricting the afferent arteriole and dilating the efferent arteriole, thus reducing the pressure inside the glomerulus.

results in a decline in both glomerular filtration rate[72] and the surface area available for filtration.[73] Decreased glomerular filtration and overt proteinuria eventually lead to renal failure.[26] Hypertension is closely related to the development of renal disease in both type 1 and type 2 patients and closely regulated blood pressure level is important in reducing renal morbidity.[74]

1.3 Insulin, Proinsulin and C-peptide

Before the discovery of insulin as a therapeutic agent for diabetes mellitus, patients were mostly treated by starvation diet; in fact death due to starvation was not uncommon among people with diabetes. In January 1922, the first insulin therapy was conducted by Fredrick Banting and Charles Best, who share the honor of discovering insulin. The patient, 14 year old Leonard Thompson, was treated successfully and lived for 13 more years until he died from pneumonia.[22(pp24)]

Insulin is secreted from the pancreatic beta cells primarily in response to glucose stimulation. It is a 51 amino acid peptide hormone, consisting of A (21 amino acids) and B (30 amino acids) peptide chains connected via two disulfide bonds. Insulin regulates glucose metabolism by increasing glucose uptake by muscle cells and adipose tissue cells, and by suppressing glucose release from the liver and kidneys.[75] In muscle and adipose tissue, insulin increases the translocation of glucose transporters (mainly Glut4) to the cell membrane, thus increasing glucose uptake. In the liver, insulin promotes glycogen synthesis by inhibiting glucose-6-phosphatase and stimulating glycogen synthase. Insulin also inhibits the release of free

fatty acids (FFA) into the circulation and promotes their clearance. FFA stimulate gluconeogenesis and decrease glucose uptake into cells. Therefore, the effect of insulin on FFA indirectly reduces glucose level in the circulation.[22 p.41]

Insulin is initially synthesized as pre-proinsulin, which transports into the rough endoplasmic reticulum, where it gets cleaved to produce proinsulin (Figure 1.4).[76] Proinsulin gets incorporated into microvesicles and travels to the Golgi apparatus, from where proinsulin containing vesicles are released. Maturation to insulin occurs inside these vesicles, as the proinsulin gets cleaved into insulin and C-peptide (connecting peptide) through the action of prohormone convertase 2, 3 and carboxypeptidase H.[77] Insulin monomers aggregate to form dimers and hexamers. Beta cell vesicles allow Zn^{2+} influx (up to mM concentration of Zn^{2+}), facilitating the formation of Zn-insulin hexamers. Zn-insulin hexamer is composed of three insulin dimers associated in a threefold symmetrical pattern with two Zn^{2+} ions along the threefold axis.[78] Importantly, C-peptide and Zn^{2+} are released along with insulin when the vesicles are released from the beta cells.

C-peptide is a 31 amino acid peptide connecting the A and B chains of insulin via dibasic amino acid links. C-peptide brings the A and B chains together in the correct orientation, so that the correct formation of disulfide bonds between the two chains is feasible.[79] Due to C-peptide release in equimolar amounts with insulin, and that it has a longer half life in the blood, C-peptide levels have been used in determining the amount of insulin release in diabetic patients. It

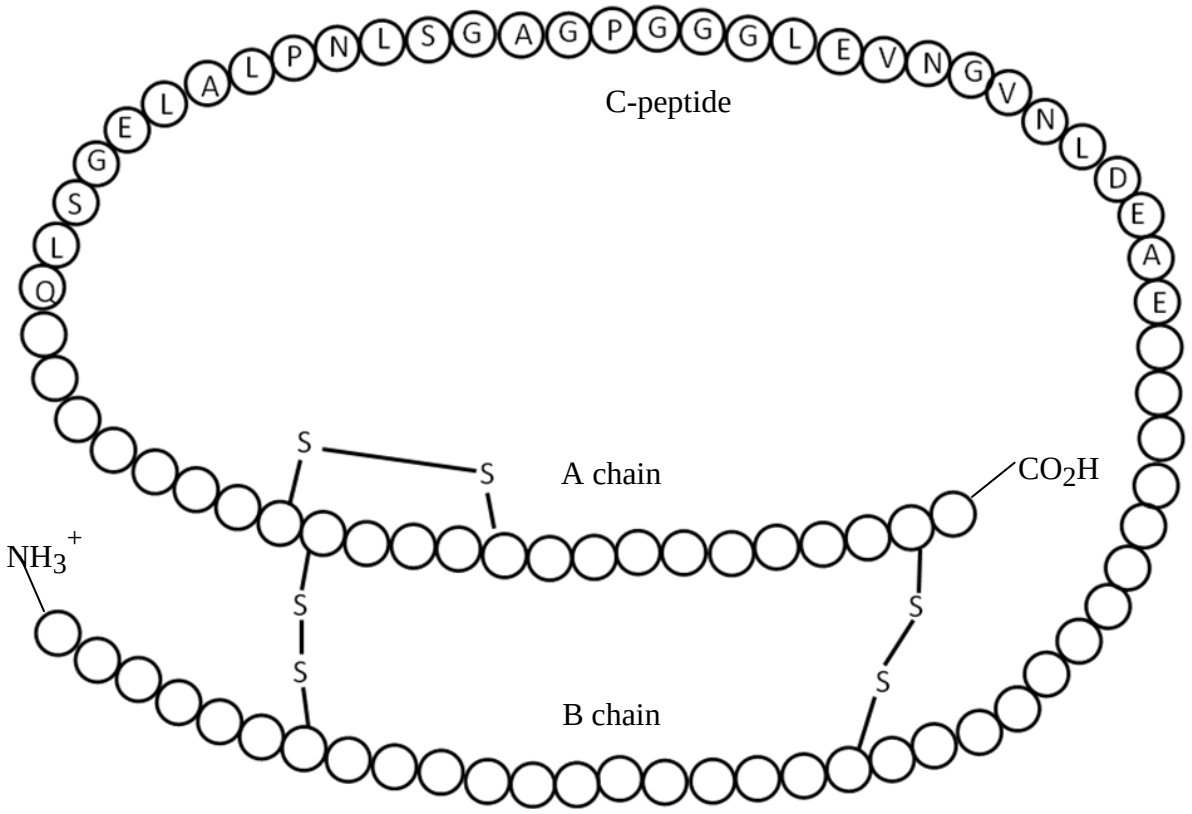

Figure 1.4: Schematic representation of the proinsulin molecule to show the A and B chains of insulin and the C-peptide. C-peptide brings the A and B chains together in the desired orientation, so that the correct formation of disulfide bonds between the two chains is feasible. The amino acids in the C-peptide are labeled in the figure.

is an important tool in determining whether the patient has type 1 or type 2 diabetes, as well as for monitoring the patient's response to drugs that stimulate insulin secretion.[80]

1.4 Role of C-peptide in Diabetic Complications

For nearly 30 years after its discovery in 1967,[81] C-peptide had been regarded as a biologically inactive peptide. However, recently, evidence has emerged indicating C-peptide might have some biological activity that is independent of insulin. There have been several reports indicating that C-peptide has beneficiary effects on diabetic complications such as neuropathy,[82, 83] nephropathy,[84, 85] retinopathy[86] and microvascular disease.[46, 87, 88]

Diabetic polyneuropathy (DPN) affects both type 1 and type 2 patients, although the progression is reported to be more rapid in type 1 patients.[55] Several attempts have been made to prevent patients from developing DPN, such as inhibiting the polyol pathway and decreasing oxidative stress, but the problem has not been sufficiently addressed.[89-91] One underlying problem of these efforts is they only focus on treating hyperglycemia in order to resolve DPN because of the assumption that the mechanism leading to DPN in both type 1 and type 2 diabetes is the same.[55, 92] In type 1 diabetes, repeated injections of insulin will decrease glucose in the circulation, but do not correct all the cellular and regulatory effects of insulin. During normal insulin secretion from beta cells, C-peptide is also released along with insulin. However, during insulin treatment, only exogenous insulin is provided for the patient. The absence of C-peptide may be a major reason for these patients to still develop neuropathy.[93]

In the nerve tissue of diabetic patients, the expression of insulin receptors (IR) becomes altered over time.[94, 95] In rat models of type 1 diabetes, C-peptide regulates the abnormalities of insulin receptor expression in the nerve tissue.[96, 97] Type 2 model rats (having circulating insulin and C-peptide) showed an insulin receptor expression similar to that of control rats.[98, 99] Decreased Na^+/K^+-ATPase activity and NO levels are two key components leading to functional nerve conduction defects.[100] At the node of Ranvier, the impaired Na^+/K^+-ATPase pump causes intra-axonal Na^+ accumulation and subsequent nodal axonal swelling and decreased transmembrane potential.[101, 102] However, C-peptide treatment dose-dependently improved the Na^+/K^+-ATPase activity, diminishing the nodal swelling and increasing the nerve conductance velocity (NCV).[83] Also in DPN, the endoneurial blood flow is decreased due to impaired expression of endothelial nitric oxide synthase (eNOS) and decreased amounts of NO in diabetic models, which causes the acute nerve conduction to slow down.[103-106] Complete C-peptide replacement in these models normalized the blood flow and vascular conductance.[103] However C-peptide had no effect on lipid peroxidation or superoxide dismutase activity, suggesting that the effects may not be due to reduced oxidative stress.

Type 1 diabetes is characterized by the down regulation of several neurotrophic factors including nerve growth factor (NGF), neurotrophin-3 (NT-3) and insulin-like growth factor-1 (IGF-1), all of which affect nerve regeneration.[96, 107, 108] C-peptide reverses this down regulation and increase the amounts of these growth factors to normal levels. However, the

reported neurotrophic effects of C-peptide were observed only in the presence of insulin, therefore, an insulin-mediated secondary effect was suggested as the cause of these effects.[93, 96] C-peptide stimulates cell proliferation in neuroblastoma cells via activation of such kinases as PI-3-kinase and p38 MAP kinase.[109, 110] The correction of neurotrophic factors and their receptors have beneficial effects on both myelinated and unmyelinated axonal size and numbers.[111, 112] The beneficial effects on nerve fiber morphology significantly improved the nerve conductance velocity and hyperalgesia (abnormally increased sense of pain).[97, 108, 113] Subcutaneous administration of C-peptide prevented and reversed hyperalgesia and enhanced firing frequency of peripheral nerves in type 1 rat models. In addition, the loss of peripheral unmyelinated fiber and atrophy of neurons involved in pain sensation, could be prevented and partially restored.[97, 108] These data suggest that C-peptide can modulate nociceptive symptoms, a common symptom in diabetic neuropathy that causes chronic pain.

Early gene responses and the expression of trophic factors (growth factor essential for the survival of the synapsing neuron) and cytoskeletal proteins were affected in nerve tissue in type 1 rat models.[107, 114] In contrast, the symptoms were considerably milder in the type 2 rat models.[98, 115] The type 2 rat models were similar in hyperglycemia to type 1 rats but had high amounts of insulin in their circulation. The suggestion for the observation was that insulin deficiency causes suppressed nerve fiber regeneration in type 1 rats.[93] However, it should be mentioned that in addition to being insulin deficient, these rats were also C-peptide deficient. C-

peptide administration into the type 1 rats prior to sciatic nerve injury resulted in immediate correction of the early gene response.[111]

Nerve regeneration requires the upregulation of neurotrophic factors in the cell somata and the availability of insulin to induce neuroskeletal protein synthesis.[93] Type 1 rats treated with C-peptide showed a timely, but slightly lower than normal, upregulation of the neurotrophic factors in the dorsal root ganglion (DRG). In axonal regeneration, a proper elongation of the axon requires the upregulation of beta-tubulin to precede the upregulation of neurofilaments. However, type 1 diabetic models lack the initial upregulation of beta-tubulin and also the necessary down regulation of neurofilaments does not occur.[111] When the type 1 models were treated with C-peptide the normal sequence and timing of the synthesis was restored, resulting in improved elongation and fiber sizes of the regenerating fibers.[111] Based on these observations, C-peptide has shown a corrective effect on regenerative capacity of type 1 diabetic polyneuropathy.[93]

For rapid nerve impulse conduction, the neuronal axon has nodes of Ranvier, periodic 1-2 μm gaps in the insulating myelin sheath covering the axon (Figure 1.5). The nerve impulse travels along the axon by 'jumping' from one node to the other. The Ranvier node contains a high density of Na^+/K^+-ATPase and Na^+-channels. One abnormality seen in type 1 diabetic models is nodal and paranodal degeneration. Interestingly, this abnormality is not seen in type 2 models having circulating insulin and C-peptide.[116-119] The abnormality occurs via the degeneration of the paranodal tight junctions attaching terminal myelin loops to the paranodal

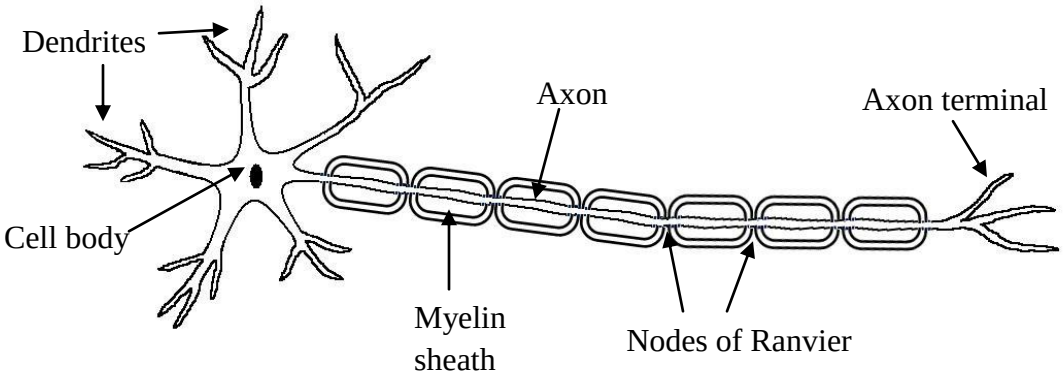

Figure 1.5: Schematic diagram of a neuron to show the Ranvier nodes. The nodes of Ranvier enable the nerve impulse to travel fast along the axon. The nodes contain Na^+/K^+-ATPase and voltage gated Na^+ channels to create the action potential. Nodal degeneration leads to impaired nerve conductance in diabetic patients.

axolemma, allowing lateralization of the nodal α-Na^+-channels,[95, 119, 120] which leads to irreversible nerve conduction defects.[95, 117, 120, 121]

One reason for paranodal dysjunction is the downregulation of adhesive proteins like contactin associated protein (caspr) and receptor-like protein tyrosine phosphatase beta (RPTB-β) that make up the tight junctions.[96, 122] Caspr is the major protein that helps make tight junction binding with other proteins like contactin, neurofascin and RPTB-β. Caspr interacts with these proteins via binding with p85 at the SH3 domain.[123, 124] P85, which is the regulatory subunit of PI3-kinase, is mediated by insulin signaling. Thus, the decrease in insulin signaling leads to the degeneration of the tight junctions as well.[96] In the presence of insulin and C-peptide these adverse effects were avoided by proper expression of the caspr, contactin, RPTP-β, and insulin receptors, and by proper binding of p85.[96] The voltage-gated-α-Na^+-channels are an essential aspect of nerve conductance and are localized at the Ranvier nodes. C-peptide stabilizes the localization of these channels via regulating expression of $ankyrin_G$ and β-Na^+-channel subunit that anchor the α-Na^+-channels at the nodal axolemma. Thus, C-peptide plays a pivotal role in keeping the paranodal structural integrity and as a result has the ability to reverse the defects in nerve conductance.[96, 112, 122, 125]

The effect of C-peptide on DPN has also been reported in experiments performed using human controls. An 80% correction of the NCV of type 1 patients having mild DPN and reduced NCV was reported during a double-blind placebo-controlled study. C-peptide was given at 1.8 mg/day for 3 months and the normalization occurred gradually and in association with normalized NCV,

these patients also showed an improvement in the perception of vibration.[126] In a larger study that included patients with advanced DPN, as well as milder DPN, a significant improvement in peak sensory NCV was observed after six months of C-peptide replacement in addition to ongoing insulin therapy. While 39% of the C-peptide group showed an improvement, only 5% of the placebo group showed the same change.[82] The patients in both studies were already on insulin therapy and these effects were not associated with improved glycemic control.[82, 126]

From the above reports it is evident that C-peptide plays a key role in ameliorating or at least reducing DPN complications. Another important neurological aspect is that type 1 diabetes, occurring in the younger population, can have adverse effects[93] on the development of the peripheral nervous system, which does not fully develop until well into the twenties.[127, 128] According to observations, insulin therapy itself is not sufficient to avoid the development of DPN; C-peptide seems to play a pivotal role in enhancing effects of insulin signaling as well as providing a sustained overall insulin effect.[122, 129-131] C-peptide also seems to have an effect in controlling blood glucose in the presence of insulin. Patients given C-peptide required smaller doses of insulin to maintain near normal glycemia.[132]

Similar to the situation in DPN, insulin therapy alone has proven to be ineffective in preventing chronic detrimental changes in renal function.[133] The addition of C-peptide therapy has shown to significantly reduce renal complications such as increased glomerular hyperfiltration,[84] increased microalbuminuria[134] and increased nitric oxide synthase (eNOS) in the afferent arteriole and glomerulus.[135] C-peptide also plays a role in protecting against

apoptosis[136] and epithelial to mesenchymal transition (EMT).[137] EMT is the trans-differentiation of tubular epithelial cells into myofibroblasts, responsible for fibrosis formation in chronic kidney disease. In diabetic patients undergoing combined kidney-islet transplantation, the success of renal transplant depends on the islet function[138] and secreted C-peptide,[139] providing basis for the assumption that C-peptide might act to protect the kidney from glycemic attack. A double-blind study of patients with type 1 diabetes and having low grade proteinuria (excess protein in urine) revealed that patients treated with C-peptide showed a 7% decrease in glomerular filtration rate (GFR) while the control patients did not show any change.[140] This experiment was to determine short term (2h) effects of C-peptide. These promising results inspired a long term study where patients with either incipient (one month long double-blind study) or mild nephropathy (three month long double-blind randomized cross-over study) were studied with C-peptide treatment. In both cases, C-peptide reduced GFR by 6% and albuminuria by 50% in comparison to patients on insulin treatment alone.[141] In rat models with streptozotocin (STZ)-induced type 1 diabetes and classic symptoms of nephropathy, C-peptide treatment decreased the GFR by 20% and albuminuria by an impressive 70% as compared to untreated control models.[85] Other studies with the same type 1 model rats have shown improved renal function as depicted by reduced GFR, decreased mesangial expansion (expansion of the membrane supporting the capillary loops in the glomerulus, Figure 1.2) and diminished albuminuria.[84, 85, 134] The role of C-peptide is not restricted to alleviating glomerular dysfunctions. As mentioned earlier, it can also exert anti-apoptotic and anti-fibrotic effects in the

proximal kidney via inhibition of tumor necrosis factor (TNF)-α induced apoptosis[136] and transforming growth factor (TGF)-β induced EMT.[137]

The relationship between circulating C-peptide and risk for vascular complications has been established during the diabetic control and complications trial (DCCT).[142] This study shows that patients with circulating C-peptide show less risk for vascular complications than patients completely lacking C-peptide. The decreased risk of microvascular disease is important because it also reduces the risk of other microvascular related diseases like neuropathy, nephropathy and retinopathy. Even in the presence of regular insulin therapy and controlled glycemic conditions, the risk for vascular disease persists in patients with diabetes, especially type 1 patients.[46] The observed effects of C-peptide on the circulation are thought to be primarily mediated via increased NO release from the endothelial cells.[87, 88, 143, 144] For example, C-peptide reduces leukocyte-endothelial interaction during endothelial dysfunction,[145] and exerts cardioprotective effects in myocardial ischemia-reperfusion in rat models.[146] It also decreases the NF-κB dependant vascular smooth muscle cell proliferation under hyperglycemic conditions.[147, 148]

In larger blood vessels, blood flow is determined by blood viscosity, vessel diameter and vessel length. In smaller vessels such as arterioles and capillaries, the elasticity of the blood cells also plays a role. Therefore, limited red blood cell elasticity will limit blood flow, if capillary diameter and pressure remain constant.[149] In diabetes mellitus patients, the red blood cell elasticity is decreased and membrane viscosity is increased contributing to red blood cell

dysfunction. This affects microvascular blood flow, and subsequent oxygen and nutrition delivery to the tissues.[42, 44, 45, 150-152]. Formation of advanced glycation endproducts (AGE), generation of free oxygen radicals and changes in ion homeostasis were attributed as causes of red blood cell dysfunction.[153] Using laser diffractoscopy, the effect of C-peptide on diabetic and non-diabetic red blood cells was investigated under shear stress.[154] Diabetic red blood cells showed significantly less deformability compared to non-diabetic red blood cells. However, C-peptide treatment restored the deformability of diabetic red blood cells while having no effect on the non-diabetic cells.[154]

Na^+/K^+-ATPase activity controls cell volume, free Ca^{2+} concentration, and membrane potential of cells.[155] Red blood cell Na^+/K^+-ATPase activity is impaired in diabetic patients, especially those with type 1 diabetes. In type 2 patients, a wide range of activity levels were observed, with patients treated with oral medication having normal activity and patients treated with insulin having lower activity.[156] This may indicate a relationship between circulating C-peptide on Na^+/K^+-ATPase activity. Patients treated with oral medication still have C-peptide secretion, while type 2 patients treated with external insulin lack C-peptide secretion. In a study with red blood cells obtained from type 1 patients, C-peptide normalized the impaired erythrocyte Na^+/K^+-ATPase activity.[157] Intravenous C-peptide treatment has also proven to improve the Na^+/K^+-ATPase activity of type 1 patients.[87]

Due to the increase in NO release from endothelial cells and increased red blood cell elasticity, C-peptide is beneficial in increasing blood flow to tissues. In isolated rat kidneys, C-peptide increased the microvascular blood flow.[158] It also evoked arterial dilation in rat skeletal muscle, an effect enhanced by the presence of insulin.[130] Diabetic rat models given C-peptide treatment twice daily for 5 weeks showed improved blood flow in anterior uvea, retina and sciatic nerve.[159] In addition, C-peptide treatment reduced permeation of albumin into the retinal and nerve tissue. The improvement in blood flow resulted in an increase in motor nerve conduction velocity. No effect could be seen in normal, healthy rats.[159] In STZ-induced diabetic rats, an improvement in nerve fiber function followed the improvement in endoneurial microvascular blood flow.[104]

During one study, C-peptide increased the forearm skeletal muscle blood flow of type 1 diabetic patients to that of the normal, healthy controls. Also, the forearm muscle cell oxygen and glucose uptake increased after C-peptide administration.[160] Transdermal iontophoresis (method used for programmed drug delivery) of C-peptide resulted in a dose-related increase in skin blood flow, similar to what was observed after iontophoresis of insulin.[143] Fernqvist *et. al.* reported that C-peptide increased the flow mediated vasodilation (FMV),[161] however, Polska et. al. observed no effect of C-peptide on retinal blood flow[162] in type 1 diabetic patients.

The effect of C-peptide largely seems to depend on the tissue type.[160, 162] Even in the same tissue/cell type sometimes there are contradictory reports on the effectiveness and the

biological activity of C-peptide.[129, 163] This might be due to the method of administrating the peptide and the concentrations that were employed during the studies. Due to its variety of actions on several types of cells, it is important to study the cellular mechanism of C-peptide in detail.

1.5 C-peptide: Structure and Molecular Mechanism

C-peptide is a 31 amino acid peptide showing no stable secondary structure in aqueous solution. In trifluoroethanol, the N-terminal third of the molecule showed an α-helical structure.[164] One much-debated hypothesis against C-peptide having biological activity is that the sequence is not conserved during evolution[165] and among species.[166] Therefore, the importance of the peptide and its sequence has been questioned. However, it should be noted that certain portions of the sequence and amino acids at certain positions have been conserved. The glutamic acid at position 1 and glutamine at position 5 in the C-terminal pentapeptide portion of C-peptide are generally conserved (~70%) in mammals.[167] According to recent work, the efficient activation of signaling pathways required the presence of conserved glutamic acid residues at positions 3, 11 and 27 and the helix promoting residues in the N-terminal segment of the peptide.[168] The pentapeptide portion of the carboxy terminal has also been proven to be essential. The rat pentapeptide (EVARQ) elicited 100% of the activity of the wild type peptide on the rat renal tube Na^+/K^+-ATPase activity.[169] The rest of the peptide evoked no activity. The human pentapeptide (EGSLQ) was able to elicit 75% of the activity of the full length peptide. Several C-peptide mid-region segments were also able to partially demonstrate the activity of the intact C-peptide, suggesting the overall picture that C-peptide structure can be divided into three

parts, with the terminal sections involved in functional interactions and the mid-region forming a joining segment.[168]

Several attempts have been made to elucidate and isolate a receptor for C-peptide, because it is believed that a receptor is necessary to elicit hormone-like effects on cells. The binding of C-peptide to pancreatic islet β-cells has been demonstrated using ^{125}I-labelled C-peptide.[170] Studies on the Na^+/K^+-ATPase activity in nerve tissue and vascular tissue using retro sequence and all D-amino acid versions of C-peptide indicated that the activity did not arise from stereo-specific binding of C-peptide to a receptor.[159] It was hypothesized that the interaction was independent of direction and chirality and consisted of poorly-defined membrane interactions. The region responsible was assumed to be the largely conserved mid-portion of the peptide, consisting of mainly non polar amino acids and a proline at position 16.

Using fluorescence correlation spectroscopy, which allows measurement of membrane interaction at the single molecule level, the binding of rhodamine-labeled human C-peptide to several different human cell membranes was studied.[171] High affinity C-peptide binding was observed with an association rate constant of approximately 3 nM, half maximal binding site occupation at 0.3 nM and full occupation at 0.9 nM. The maximum number of binding sites per cell was found on renal tubular cells at approximately 1000-1500 per cell. The binding could be displaced by excess amounts of C-terminal pentapeptide and intact C-peptide, but not by scrambled C-peptide, insulin, insulin like growth factor (IGF)-I or IGF-II. The binding could be largely inhibited by pertussis toxin (PTX) pre-treatment of the cells, indicating the binding may be through a G-protein coupled receptor (GPCR) for C-peptide, linked to the G-protein α-subunit

(either $G\alpha_i$ or $G\alpha_o$). However, a receptor for C-peptide has not been isolated via gene-cloning strategies or proteomics studies. The attempts to isolate a receptor by screening a human lung fibroblast λphage cDNA expression library and by proteomic analysis of proteins co-immunoprecipitated from C-peptide treated human fibroblasts using anti-C-peptide antibodies were reported unsuccessful.[172] This does not mean a receptor for C-peptide is non–existent; with different approaches a receptor may be identified and isolated in the future.

The total mechanism of C-peptide bio-activity is not yet fully understood. However, the effect of C-peptide on Na^+/K^+-ATPase activity, endothelial NO synthase, mitogen activated protein kinase (MAPKs) pathway, PI-3-kinase pathway and transcription factors have been described. Na^+/K^+-ATPase is a membrane associated protein complex that uses ATP hydrolysis to drive the counter transport of Na^+ and K^+ across the cell membrane. In diabetes, impaired Na^+/K^+-ATPase activity can be observed in various cell types such as peripheral neurons, red blood cells and renal tubular tissues, and it can lead to many diabetic complications.[173-176] It has been well demonstrated that C-peptide has the ability to regulate the Na^+/K^+-ATPase activity. Low circulating C-peptide concentration has been related to decreased Na^+/K^+-ATPase activity in red blood cells.[156] In rat kidney tubules, C-peptide activated this enzyme at a dose range of low to high nM concentrations, and the effect was diminished by PTX, indicating the dependency on a GPCR-related pathway. Treatment with a Ca^{2+}-calmodulin dependant protein phosphatase 2B (calcineurin) inhibitor, FK506, also abolished the C-peptide effect. The observations suggest that C-peptide might increase the Na^+/K^+-ATPase activity by binding to a

GPCR and activating a Ca^{2+} dependant signaling pathway.[177] C-peptide was found to activate the Na^{+}/K^{+}-ATPase in isolated rat kidney medullary thick ascending limb tubules (MTALs) at physiological concentrations.[178] The treatment resulted in phosphorylation of the Na^{+}/K^{+}-ATPase α-subunit and translocation of the Ca^{2+} dependant protein kinase C (PKC-α) to the membrane, thus activating PKC-α. Both of these effects were blocked by an inhibitor of PKC. Overall, these experiments are indicative of a signaling pathway of C-peptide mediated by intracellular Ca^{2+} and PKC, again suggesting the presence of a GPCR for C-peptide.[167] Vasopressin is another stimulating molecule for Na^{+}/K^{+}-ATPase activity. In human skin fibroblasts and mesangial check spelling cells, C-peptide increased the expression of the vasopressin-activated calcium mobilizing receptor (VCAM-1) at both RNA and protein levels.[179] The effect was optimal at a concentration of 1 nM C-peptide and was inhibited by PTX. This upregulation of vasopressin activity may indicate another mechanism for C-peptide induced increase in Na^{+}/K^{+}-ATPase activity .[167]

In a study with diabetic red blood cells, C-terminal pentapeptide and hexapeptide portions of C-peptide improved the diabetic red blood cell deformability similar to the intact peptide. However, the middle segment did not have any effect.[180] To evaluate the signaling pathway the cells were pre-treated with ouabain, EDTA and pertussis toxin (PTX). Ouabain inhibits Na^{+}/K^{+}-ATPase, EDTA removes Ca^{2+} from the medium and PTX inhibits the G-protein coupled receptor (GPCR) pathway. Only Oubain and EDTA had an effect on the C-peptide induced red blood cell deformability, suggesting that Ca^{2+} influx was required for Na^{+}/K^{+}-

ATPase activity. However, C-peptide did not mediate the effect via a GPCR during this interaction.

Another beneficiary effect of C-peptide is its ability to increase microvascular blood flow, which is often reduced in diabetes. The mechanism responsible for increased blood flow is suggested to be mediated via increased nitric oxide (NO) production. NO is a known smooth muscle relaxing agent and a vasodilator that is produced in endothelial cells via activation of endothelial NO synthase (eNOS). NO released by endothelial cells stimulates guanylcyclase in smooth muscle cells. This, in turn, increases the production of cyclic GMP (cGMP) in smooth muscle cells, causing vasodilation.[181-184]

The first observation suggesting that C-peptide stimulates NO release was that the inhibition of NO synthase (NOS), by administration of a NOS inhibitor, had an effect on the C-peptide induced increase in glucose utilization of type 1 model rats.[185] It has also been demonstrated that C-peptide mediated arteriolar dilation is dependent on NO.[130, 186] In the study, C-peptide induced a vasodilatory effect in isolated rat cremaster muscle arterioles that could be inhibited by N(omega)-nitro-L-arginine (L-NNA), which is a known NOS inhibitor. However, this effect was seen only in the presence of insulin.[130] A study conducted on the effect of C-peptide on bovine aortic endothelial cells (BAEC) reported that at physiological postprandial (after a meal) concentrations, C-peptide induced NO release from these cells and the effect was associated with an increase in intracellular Ca^{2+} levels.[187] C-peptide also increased the Ca^{2+} influx to the renal tubular cells.[169] The NO production by C-peptide in BAEC was completely abolished by L-NNA and the presence of EDTA, a strong metal chelator, in the cell

culture medium. These observations suggest that C-peptide induced NO release from endothelial cells is eNOS mediated and Ca^{2+} dependant.[187]

Another observation of the effect of C-peptide on NOS was that in BAECs, C-peptide upregulated the eNOS gene transcription, indicating that it could increase the NOS expression.[188] This effect was through phosphorylation and activation of extracellular signal-regulated mitogen activated protein kinase (ERK).

C-peptide has been shown to trigger phosphorylation and activate several proteins in the mitogen activated protein kinase (MAPK) family. MAPK family is constituted of many proteins including ERK-1 and 2, c-Jun N-terminal kinases (JNK) and p38s (E.g. p38α, p38β).[167] Activation of ERK in response to C-peptide treatment has been observed in different cell types such as mouse embryonic fibroblast cells (cell line Swiss 3T3),[189] mouse lung capillary endothelial cells,[144] opossum kidney immortalized proximal tubular cells[190] and bovine aortic endothelial cells.[188] However it has not been observed in other cell lines such as 3T3-L1 cells, L_6E_9 muscle cells, and $HepG_2$ hepatoma cells. The ability and optimal concentration of C-peptide to show the activation largely depends on the cell type. In cells such as LEII mouse lung capillary endothelial cells C-peptide activated both ERK and p38. Consequently, p38 led to the activation of cAMP response element binding protein (CREB) and activating transcription factor -1 (ATF-1), causing these transcription factors to bind to CRE (cAMP response element) in DNA and thereby regulate gene transcription.[188] In opossum kidney cells, C-peptide caused an influx of Ca^{2+} and a consequent translocation and activation of PKCα.[190] As mentioned earlier

PKCα is reported to activate Na^+/K^+-ATPase in kidney tubular cells.[178] Interestingly, all of these effects were sensitive to PTX, indicating the presence of a GPCR for C-peptide.[167]

Another recently discovered molecular effect of C-peptide is its ability to stimulate the translocation of the GTP binding protein (Rho A) from the cytosol to the cell membrane. This effect was observed in human kidney proximal tubular cells.[191] In these cells, the activation of ERK, JNK, PKCε, PKCδ and the translocation of Rho A were sensitive to inhibition of phospholipase C (PLC), indicating an upstream PLC activation by C-peptide responsible for these effects. Again, all effects were inhibited by PTX.[191]

Looking at all the reports, the MAPK activation of C-peptide can be summarized as follows: i) Binding of C-peptide to a GPCR which can be inhibited by PTX; ii) activation of PLC; iii) Ca^{2+} influx and subsequent increase in intracellular Ca^{2+} and diacylglycerol levels stimulate several PKC isoforms; iv) PKC dependent activation and translocation of Rho A and phosphorylation and activation of MAPKs.[167]

Another family of proteins activated by C-peptide is the phosphatidylinositol-3-kinases (PI-3-kinases), which phosphorylate the hydroxyl group at position-3 of the inositol ring in phosphotidylinositol. PI-3-kinases are a key component in the insulin signally pathway as well, and they stimulate a variety of cellular functions such as growth, proliferation, survival, differentiation and also intercellular trafficking. PI-3-kinases mediate most of these functions via their ability to activate protein kinase B (Akt).[192] C-peptide activation of PI-3-kinase has been demonstrated in various types of cells, including opossum kidney cells,[190] Swiss 3T3

fibroblasts,[189] human $CD4^+$ T cells,[193] SHSY5Y neuroblastoma cells,[110] and L6 myoblasts.[129] C-peptide induced activation of PI-3-kinase can lead to enhanced neuronal and renal tubular cell proliferation,[110, 190] increased T-cell migration,[193] increased peroxisome proliferators activated receptor-γ (PPARγ) stimulation in kidney tubular cells and subsequent gene transcription,[194] and upregulated glycogen synthesis in muscle cells.[129]

One downstream effect of MAPK and PI-3-kinase activation is the activation of transcription factors, which regulate gene and protein expression. In mouse lung capillary endothelial cells, C-peptide activates transcription factors CREB and ATF 1,2 proteins that can bind to specific response elements such as CRE in DNA and regulate gene expression.[144] Interestingly the activation of the CREB and ATF proteins are inhibited by the p38 inhibitor SB203580 showing that it is a downstream effect of p38 activation. Enhanced expression and translocation of nuclear factor-κB (NF-κB), which governs antiapoptotic effects, have been observed in neuroblastoma cells and Swiss 3T3 cells after C-peptide treatment.[110, 195] It should be mentioned that both insulin and C-peptide have the ability to activate PI-3-kinases, therefore both could have activated transcription factors such as NF-κB and PPARγ in opossum kidney tubular cells.[136] However, PTX inhibited only the activation by C-peptide, indicating a different signal pathway for C-peptide than that of insulin. Because MAPK and PI-3-kinase activation is also a downstream effect of insulin signal pathway, it has been debated whether C-peptide activates the insulin signaling receptors. There are data supporting[129] and opposing this idea.[136, 196] However, taking into consideration that only the C-peptide effect is inhibited by PTX, and

that C-peptide provokes GTPγS binding to $G\alpha_i$,[136] which is typical for a GPCR binding behavior, and also that plasma resonance based data indicates that there is no binding between C-peptide and purified insulin receptor A or insulin-like growth factor receptor.[197] Thus, C-peptide seems to be following a different signaling pathway.

Considering the molecular mechanisms of C-peptide, it can be concluded that most of the favorable effects of C-peptide in reducing diabetic complications can be explained by its ability to activate eNOS, enhance Na^+/K^+-ATPase activity, trigger signaling pathways like MAPK or PI-3-K, and activate transcription factors. All of these mechanisms are aided by the presence of ATP. Therefore, one hypothesis that can be considered is, C-peptide can increase cellular metabolism resulting in increased production and release of ATP from cells. Therefore, it is a possibility that the initial effect of C-peptide, at least in most cells, is an increase in cellular metabolism.

Johansson *et. al.* have shown that in type 1 patients treated with C-peptide for two 60 min periods, the glucose utilization increased by 25% compared to that of control patients treated with NaCl.[140] In a follow-up study, after one month of C-peptide administration, the glycated hemoglobin (HbA1C) level in type 1 patients decreased by 9-16%, showing C-peptide improves glycemic control.[141] Both these patient groups were already under insulin treatment when C-peptide was administered. Using surface plasmon resonance (SPR) and electrospray ionization mass spectrometry (ESI-MS) studies, Shafqat et. al. demonstrated that C-peptide helps in disaggregation of the Zn-insulin hexamer.[132] They also showed that when insulin and C-peptide

were administered together, the patients developed 66% more stimulation of glucose metabolism than when given insulin alone.

Recent findings in the Spence group have shown that C-peptide increases the ATP release from red blood cells, but only in the presence of a metal ion such as Cr^{3+}, Zn^{2+} or Fe^{2+}.[198, 199] ATP is a known stimulus for endothelial NO production. In this regard, ATP released from red blood cells can be a determinant of improved blood flow, and can participate in the reduction of the microvascular complications of diabetes. One observation supporting this theory is that in the presence of red blood cell-derived ATP, platelet adhesion to an endothelium decreases by approximately 50%.[200] This suggests that red blood cell derived ATP plays a major role in affecting vascular complications like platelet-endothelial interaction. This may not be true for all cell types, but we have seen C-peptide induced ATP release from, and glucose uptake in to, red blood cells and well as endothelial cells, both of which cell types that do not have insulin sensitive glucose transporters.

The C-peptide induced ATP release was only seen when a metal ion such as Cr^{3+} or Fe^{2+} was present, showing the necessity of metal-activation for proper C-peptide activity.[198] However, Zn^{2+} is the most abundant metal ion in the pancreas, and therefore has the highest potential to interact with C-peptide *in vivo*. Therefore, later studies were performed with Zn^{2+} as the metal ion instead of Cr^{3+} or Fe^{2+}. Red blood cells obtained from a type 1 rat model showed same percent increase in ATP release when stimulated with Zn^{2+}-activated C-peptide, as that of healthy red blood cells. However, in red blood cells obtained from a type 2 rat model, Zn^{2+}-

activated C-peptide stimulated less ATP release than that of normal red blood cells, showing some kind of resistance of these cells to C-peptide.[199] Pre-treatment of these cells with metformin, a common medication for insulin resistance in diabetic type 2 patients, restored C-peptide induced ATP release to that of the normal red blood cells. This suggests that type 2 patients can show not only insulin resistance, but also C-peptide resistance.

Observing that a metal was needed for C-peptide activity, it is possible that the inhibitory effect of EDTA on C-peptide action in some studies[180, 187] could also be the result of 'inactivation' of C-peptide due to complexation of metal ions by EDTA. It also suggests that success of clinical studies can depend on the way C-peptide is handled, i.e. whether a metal ion is present or not, thus explaining C-peptide's poor reproducibility in some studies. Also, in some studies where simultaneous insulin administration was needed to either induce or increase C-peptide activity,[96, 129] Zn^{2+} association with the insulin hexamer may have 'activated' the C-peptide, leading to positive results. For example, in the study by Shafqat *et. al.*, when insulin and C-peptide were administered through the same port to the subject, the decrease in glucose level was significantly higher than in separate administration of insulin and C-peptide via different ports.[132] The authors credited this observation to the ability of C-peptide to break down the Zn-insulin hexamer. However, another possibility is that, during the process of administering insulin and C-peptide together, C-peptide has the ability to get 'activated' by Zn^{2+} that is always present in the insulin sample.

Although many beneficial effects of C-peptide have been reported, there is still doubt about using C-peptide as a therapeutic agent along with insulin. The drawbacks pointed out are:

i) a cellular receptor has not been identified for C-peptide, ii) long-term successful clinical studies have not been reported, iii) even in the presence of C-peptide, patients with type 2 diabetes develop complications of diabetes mellitus (these patients have circulating insulin and C-peptide).[172] Although most of these questions can be answered with existing results, further investigations are needed to firmly establish C-peptide as a therapeutic agent for complications of diabetes mellitus.

The aim of this dissertation is to improve our understanding about the necessity of a metal ion for biological function(s) of C-peptide, provide further evidence of Zn^{2+} binding to C-peptide, investigate the effect of metal-binding on C-peptide interaction with red blood cells, and suggest a possible route to determine a cellular receptor for C-peptide, validating the potential of C-peptide as a therapeutic agent in future diabetes treatment.

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Chapter 2 - Investigation of a Possible Mechanism of Zn^{2+} Activation of C-peptide

2.1 The β-cell Granule and Secretion of Insulin

The pancreas has both endocrine and exocrine tissue. The endocrine tissue, composed of islets of Langerhans, is scattered throughout the exocrine tissue. In an adult pancreas the islets of Langerhans constitute 1-2% of the pancreatic mass, approximately two million clusters of cells. The islet cells are of four different types; α-cells, β-cells, δ-cells and pancreatic polypeptide (PP) cells. β-cells are responsible for synthesizing, storing and secreting insulin.[1]

As mentioned in the previous chapter, insulin is first synthesized as preproinsulin in the cytosol of the β-cell and is internalized into the rough endoplasmic reticulum (RER) where it is cleaved by proteolytic enzymes, removing the signal peptide to form proinsulin. Proinsulin consists of the A and B chains of insulin connected via the C-peptide.[2] Proinsulin is then transported to the Golgi apparatus with the aid of microvesicles,[3] and from the there it is packaged into vesicles. The membrane of these vesicles contains an ATP-dependant proton pump that regulates the pH inside the vesicle.[4] The conversion of proinsulin to insulin starts in the Golgi complex and continues in the maturing vesicles. Two endopeptidases (prohormone convertases 2 and 3) and carboxypeptidase H sequentially act to remove the C-peptide from proinsulin, yielding insulin.[5]

Another change that takes place during granule maturation is the efflux of Zn^{2+} ions into the granule via the Zn^{2+} transporter (ZnT-8) in the granule membrane.[6] Thus, the concentration

of Zn^{2+} inside the granule approaches mM levels.[4] Proinsulin forms a hexamer combining with Zn^{2+} ions (two Zn^{2+} ions per hexamer),[7] and when the C-peptide is cleaved, the resulting Zn-insulin hexamer complex, which has lower solubility than Zn-proinsulin, forms microcrystals inside the secretory granules.[8] Insulin is stored in the crystalline form until the mature granules are released by exocytosis in response to stimulation, typically an increase in glucose metabolism in the cell (Figure 2.1). Increased blood glucose levels (> 5.5 mM glucose) result in increased glucose transport into the β-cell through the glucose transporter GLUT-2. This allows for rapid equilibration between intracellular and extracellular glucose concentrations. Once inside the cell, glucose is phophorylated by glucokinase. This is the rate limiting step in glucose metabolism, hence glucokinase acts as the 'glucose sensor' in β-cells, coupling insulin secretion to outside glucose concentration.[9]

Increased glucose metabolism elevates the ATP/ADP ratio in the β-cell. Increased ATP closes the ATP sensitive K^+ channels leading to a depolarization in the cellular membrane,[10] which, in turn, opens the voltage gated Ca^{2+} channels increasing the Ca^{2+} influx to the cell.[11] The increase in intracellular Ca^{2+} leads to β-cell granule exocytosis.[12, 13] Insulin and C-peptide are released in equimolar amounts during this exocytosis, and the insulin hexamer breaks down into monomers, which are the active form of the hormone, thereby releasing the bound Zn^{2+}.[6] The insulin hexamer shows a high binding affinity for Zn^{2+} ($K = 1 \times 10^9$),[14] while the insulin monomer has a lower binding affinity ($K = 1.86 \times 10^5$).[15]

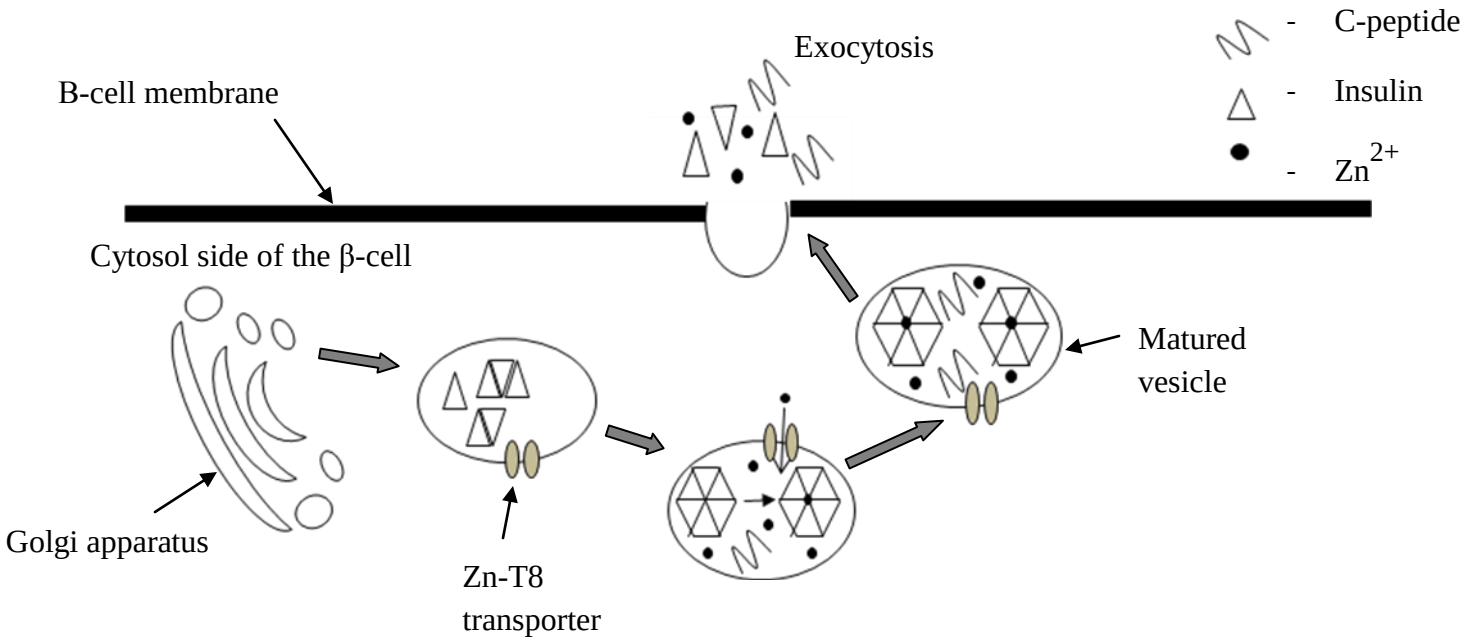

Figure 2.1: Maturation and exocytosis of beta cell granules. Zn^{2+} enters the granule through ZnT-8 transporters. During exocytosis, insulin, C-peptide and Zn^{2+} are released simultaneously into the extracellular space.

2.1.1 Change of pH Inside the β-cell Granule and Its Effect on Insulin Secretion

pH plays a major role in controlling the synthesis and storage of insulin. It has been reported that the pH inside the secretory granules decreases with maturation, yielding mature granules with an internal pH between 5 and 6.[16, 17] The acidification is needed for the proteolytic processing of proinsulin, because the activities of the proteolytic enzymes involved are pH dependent.[18-20] The low pH also favors the storage of the insoluble Zn-insulin hexamers.[17] During exocytosis, the interior of the granule undergoes a rapid pH change from ~5.5 to 7.4. This pH change is necessary for the dissolution and dissociation of the Zn-insulin hexamer.[21] Kennedy *et. al.* observed that when the extracellular pH was kept at 7.4 during exocytosis of the granules, free insulin (dissociated from Zn^{2+}) was detected. However, at pH 6.4 no free insulin could be detected. Using surface plasma resonance (SPR) and ESI-MS, the ability of C-peptide to increase Zn-insulin hexamer dissociation has also been demonstrated, although the molecular mechanism of this process has not been explained.[22]

2.1.2 Role of Zn^{2+} in Insulin Synthesis, Storage and Secretion

Zinc is an essential trace element found in a variety of proteins, metalloenzymes and transcription factors.[23] It is also believed to play a vital role during insulin synthesis.[8] The concentration of Zn^{2+} in the pancreatic β-cell is among the highest in the body (~20 mM).[24] Zn^{2+} in the beta cell granules serves several functions during insulin biosynthesis: assembly of the proinsulin hexamers, increasing solubility of proinsulin hexamer, and crystallization of

insulin hexamers.[25] Formation of Zn-insulin crystals is believed to protect the insulin molecule from proteolytic enzymes.[26]

Studies with ZnT-8 knockout mice (unable to produce the ZnT-8 transporter) have shown that Zn^{2+} is important for insulin storage as the Zn-insulin hexamer and for insulin transcription and processing. The knockout mice showed lower conversion of proinsulin to insulin, due to a decrease in the expression of proinsulin converting enzymes. Therefore, during exocytosis, these mice released lower amounts of biologically active insulin than normal controls.[27] Zinc deficiency has been linked to the progress of both type 1 and type 2 diabetes.[28, 29] Under certain conditions, such as STZ-induced diabetes mellitus in rats, Zn^{2+} can cause islet cell death.[30] This adverse effect can be avoided by chelation of Zn^{2+} with a mild complexing agent such as clioquinol.[31]

Meyer et. al. first reported the necessity of the presence of a metal ion for proper function of C-peptide, in stimulation of ATP release from red blood cells.[32] The metal ions used in this study were Fe^{2+} and Cr^{3+}. However, as discussed above, Zn^{2+} is abundant in the pancreatic β-cells and has the potential to activate C-peptide. Therefore later studies were done using Zn^{2+} with C-peptide and similar successful results were obtained.[33] The mechanism behind this metal-activation and role of the metal ion during the stimulation of ATP release will be discussed in this chapter.

2.2 Experimental

2.2.1 Preparation of Reagents

Purified water with 18.2 MΩ resistance (Easypure® II ultrapure water system, Barnstead) was used for all experiments to ensure absence of metal ion contamination. All reagents were prepared and stored in polypropylene tubes whenever possible and any glassware used was acid-washed prior to use to avoid any metal-ion contamination.

Zn^{2+} stock solution was prepared by dissolving 5.5 mg of zinc (II) chloride (Jade Scientific, Canton, MI) in 500 mL of purified water to obtain a final concentration of 80 µM. Tris buffer was made by dissolving 0.1211 g Trizma base (2-Amino-2-(hydroxymethyl)-1,3-propanediol) (Sigma-Aldrich, St. Louis, MO) in water to give a 10 mM solution followed by dropwise addition of 0.2 M HCl to reach a final pH of 7.4. MES buffer was made by dissolving 0.0976 g 2-(N-Morpholino)ethanesulfonic acid (Sigma-Aldrich, St. Louis, MO) in water to give a 10 mM solution followed by the dropwise addition of 0.2 M NaOH to reach a final pH of 5.5. Buffers were made fresh before each experiment to minimize dissolved bicarbonate (HCO_3^-), while pH-verified purified water was collected immediately before use the experiments.

TSQ (N-(6-Methoxy-8-quinolyl)-p-toluenesulfonamide) (AnaSpec, Fremont, CA) stock solution was prepared by dissolving 1 mg of the solid in 3 mL of dimethylsulfoxide (DMSO) to give a final concentration of 1 mM. The stock solution was stored in the dark at 4 ºC until use.

2.2.2 Purification and Characterization of C-peptide, and Mutants of C-peptide, Using High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS)

Crude C-peptide (Genscript, Piscataway, NJ) and its single amino acid mutant peptides (Table 2.1) (synthesized by Zachary Keltner, Reid Lab, Michigan State University, MI) were purified using reverse phase high performance liquid chromatography (RP-HPLC) with a Shimadzu (Columbia, MD) LC-20AB solvent system and a SPD-20AV UV-Vis absorbance detection system. 20 mg of each peptide were dissolved in a minimal amount of water (about 4 x 1 mL) and up to 10% of acetonitrile (ACN) in cases where the peptide had lower solubility. Samples were injected on to the column, a Vydac C18 Protein and Peptide column (Grace, Deerfield, IL), (25 cm column length, 5 µm particle size and 5 mm i.d.) with a 2 mL stainless steel loop connected to a Rheodyne injection valve (model 9215, Oak Harbor, WA). To obtain optimal separation of crude peptides, two separate multi-segment gradients were used (Tables 2.2 and 2.3) for C-peptide and mutants peptides. The flow rate used was 5 mL/min. Solvent A was 0.1% HPLC-grade trifluoroacetic acid (TFA) (Pierce, Rockford, IL), in 18.2 MΩ resistance water purified using a Nanopure Diamond Filtration system (Dubuque, IA). Solvent B was 0.089% TFA in 60% HPLC-grade ACN (EMD Biosciences, Gibbstown, NJ). The solvents were purged with helium at 10 psi for 15 minutes prior to running the HPLC. The eluting peptides were monitored at 215 nm, and 5 mL fractions were collected into 15 mL polypropylene tubes (Greiner Bio-One, Monroe, NC).

The fractions were analyzed for purity using LC-MS and MS/MS. The analyses were performed using a Thermo LTQ linear quadrupole ion trap mass spectrometer (Thermo, San

C- peptide mutant	Amino acid sequence
Wild type	EAEDLNVGNVELGGGPGAGSLNPLALEGSLQ
E1A	**A**AEDLNVGNVELGGGPGAGSLNPLALEGSLQ
E3A	EA**A**DLNVGNVELGGGPGAGSLNPLALEGSLQ
D4A	EAE**A**LNVGNVELGGGPGAGSLNPLALEGSLQ
E11A	EAEDLNVGNV**A**LGGGPGAGSLNPLALEGSLQ
E27A	EAEDLNVGNVELGGGPGAGSLNPLAL**A**GSLQ

Table 2.1: Amino acid sequences of C-peptide and its single amino acid mutants.

Time (min)	%B
0	0
5	40
30	50
40	100
45	100
50	0
55	stop

Table 2.2: Multi-segment gradient used for purification of wild type C-peptide. Solvent A - 0.1% trifluoroacetic acid (TFA), Solvent B - 0.089% TFA in 60% ACN.

Time (min)	%B
0	0
20	34
100	67
120	100
125	100
130	0
135	stop

Table 2.3: Multi-segment gradient used for purification of the C-peptide mutants. Solvent A - 0.1% trifluoroacetic acid (TFA), Solvent B - 0.089% TFA in 60% ACN.

Jose, CA) equipped with an Advance nanoelectrospray source and Paradigm MS4 capillary RP-HPLC system (Michrom Biorsources, Auburn, CA). The RP-HPLC column was a 200 µm i.d. x 50 mm fused silica column packed with Magic C4 (5 µm; Michrom Bioresources, Auburn, CA). Samples were injected from a Paradigm AS1 autosampler (Michrom Bioresources, Auburn, CA) at a flow rate of 2 µL min^{-1} and the peptides were eluted using a linear 55 min gradient from 95% solvent A (0.1% formic acid in water) to 60% solvent B (0.1% formic acid in ACN). The ion transfer tube of the mass spectrometer was set at 180 ºC and the spray voltage was maintained at 2.0 kV. The activation time for MS/MS was maintained at 30 ms using an activation q value of 0.25. The isolation window was maintained at 2.0 *m/z* while the normalized collision energy was set at 35. The spectra were recorded in centroid mode. Analyses were performed using Xcalibur software (Thermo, San Jose, CA).

After the LC-MS analyses the fractions containing pure peptide were combined and lyophilized overnight. The dried peptides were weighed and stored at -20 ºC until further use. No further purification of the fractions was performed. However, because the peptides were purified using the same method and solvents, any impurities present in the purified fractions were considered equivalent. Further studies with C-peptide, solvent exchange to remove TFA is recommended. However, experiments have shown that the TFA had no effect on C-peptide/Zn^{2+} binding.

2.2.3 Determination of Binding Constant and Stoichiometric Ratio Between Zn^{2+} and C-peptide Using Fluorescence Spectroscopy

All fluorescence measurements were performed using a FluoroMax®-4 Spectrofluorometer (HORIBA-Jobin Yvon, Edison, NJ) with a 500 µL quartz cell having a 1 cm path length. 10 mM Tris buffer was used for samples prepared at pH 7.4, while 10 mM MES buffer was used for samples prepared at pH 5.5. To prepare a sample for analysis, C-peptide and Zn^{2+} (as $ZnCl_2$) were mixed in water (pH ~ 5.5) in various concentrations. After 2-3 minutes, the appropriate buffer was added, followed immediately by TSQ. After 45 s, the fluorescence emission was measured at 480 nm (excitation at 362 nm with an excitation slit width of 2 nm and an emission slit width of 3 nm). The Zn^{2+} concentration in the final solution was varied from 0.1 µM to 0.6 µM in 0.1 µM increments, while the C-peptide concentration was kept at 0.1 µM. The TSQ concentration used was 1.5 µM in each solution. TSQ was needed in excess to ensure that all free Zn^{2+} was detected, even in the sample containing the highest amount of Zn^{2+} (0.6 µM Zn^{2+}), and to keep it constant in all solutions. The water content of each sample was kept constant to avoid dilution of the buffer. After calibration with standards, the fluorescence signal could be used to determine the concentration of Zn^{2+} bound to the peptide and unbound Zn^{2+} in the sample. The binding curves were plotted and the data was linearized using Scatchard analysis.[34] From the Scatchard plots, the stoichiometric ratio between Zn^{2+} and C-peptide was determined.

To determine the effect of the solvent used for mixing, C-peptide and Zn^{2+} were also mixed in Tris or MES instead of water. TSQ was added immediately after and the fluorescence signal was measured as described above. All other conditions were the same as those listed above. The binding of C-peptide mutants were determined by repeating the experiments with substitution of C-peptide with the relevant mutant.

2.2.4 Circular Dichroism (CD) Studies to Determine Structural Changes in C-peptide with the Addition of Zn^{2+}

All studies were performed on a Chirascan CD spectrometer (Applied Photophysics Limited, Leatherhead, UK) using a quartz cell having a pathlength of 0.1 cm. The C-peptide concentration used for all measurements was 20 µM. C-peptide and Zn^{2+} were mixed in water and brought up to 500 µL with the buffer (10 mM Tris, pH 7.4) and the spectrum was immediately obtained. In samples where only C-peptide was present, the same amount of water was added in place of the Zn^{2+}. The far UV region (190 nm to 260 nm) was measured and data points were taken at 1 nm increments with a 2.5 s averaging time. A buffer spectrum was subtracted from each spectrum. Each spectrum reported is an average of 3 obtained spectra for separate preparations. All measurements were taken at room temperature (~ 24 ºC).

2.3 Results

RP-HPLC and ESI-MS were used to purify and characterize the C-peptide and its mutants. The chromatograms from HPLC show resolution of the peptide peaks (Figures 2.2 and 2.3), while the mass spectra validate the presence/absence of the peptide in each fraction (Figures

2.4 and 2.5). To study the binding between C-peptide and Zn^{2+}, the fluorescence probe TSQ (N-(6- methoxy-8- quinolyl)-p-toluenesulfonamide) was used to generate a measurable signal. TSQ (Figure 2.6) was selected for the study due to its high selectivity and moderate binding affinity (1.02×10^6 M^{-1} in 10 mM Tris) for Zn^{2+}.[35] When excited at 362 nm, TSQ bound to Zn^{2+} gave a fluorescence emission peak with a maximum around 485 nm. In all the studies described, the emission at 485 nm was used for data analysis. When C-peptide was present, the emission intensity decreased (Figure 2.7). This suggests that less Zn^{2+} was available for binding with TSQ, and given that there were no other species present in the solution to bind with Zn^{2+}, it was assumed that the remaining Zn^{2+} was bound to C-peptide. To generate data for Scatchard analysis, the C-peptide concentration was kept constant while the Zn^{2+} concentration was increased to reach a 1:6 ratio of C-peptide to Zn^{2+} ions in the medium. At each concentration of Zn^{2+}, the amount of bound Zn^{2+} and free Zn^{2+} was calculated with the aid of a calibration curve (Figure 2.8 and Table 2.4). The binding data were represented using Scatchard plots (Figure 2.9).

Scatchard plots have been considered a suitable means of data representation in binding systems.[36] At equilibrium for each system containing one single set of binding sites, the Scatchard plot gives a straight line according to the following equation:

$$r / C_{L(f)} = (n - r) K \qquad (1)$$

or, if the concentration of the binding component (protein) is unknown:

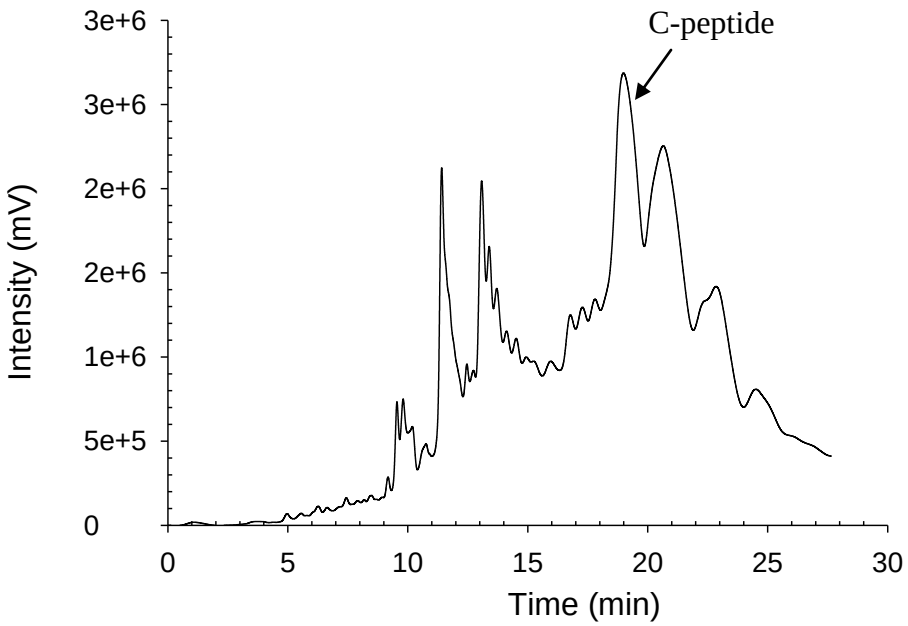

Figure 2.2: Purification of crude C-peptide by RP-HPLC. 20 mg of peptide were purified using a multi-segment gradient. The peptide fractions were detected at 215 nm. C-peptide started eluting at 19.0 min.

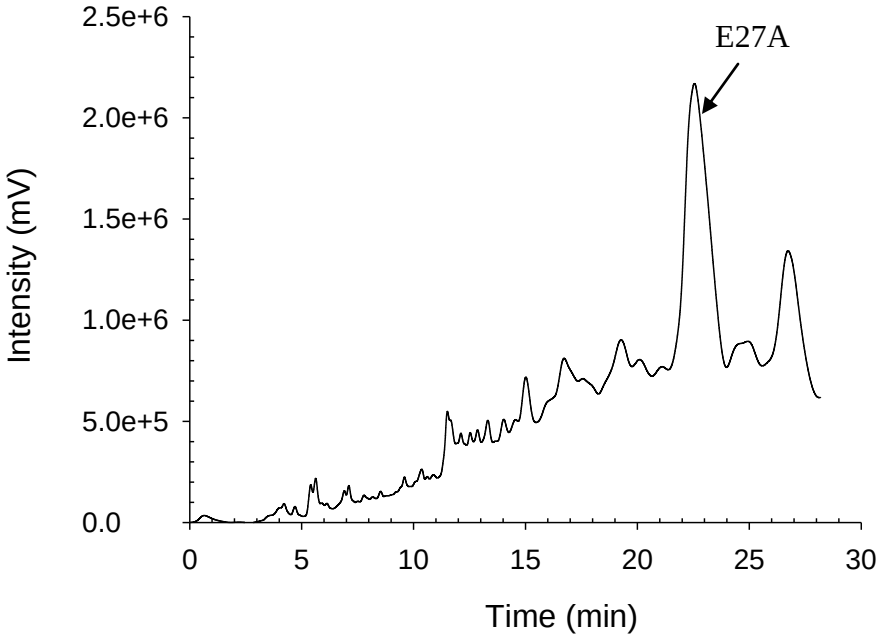

Figure 2.3: Purification of crude C-peptide mutant E27A by RP-HPLC. 10 mg of peptide were purified using a multi-segment gradient. The peptide fractions were detected at 215 nm. E27A started eluting at 22.0 min.

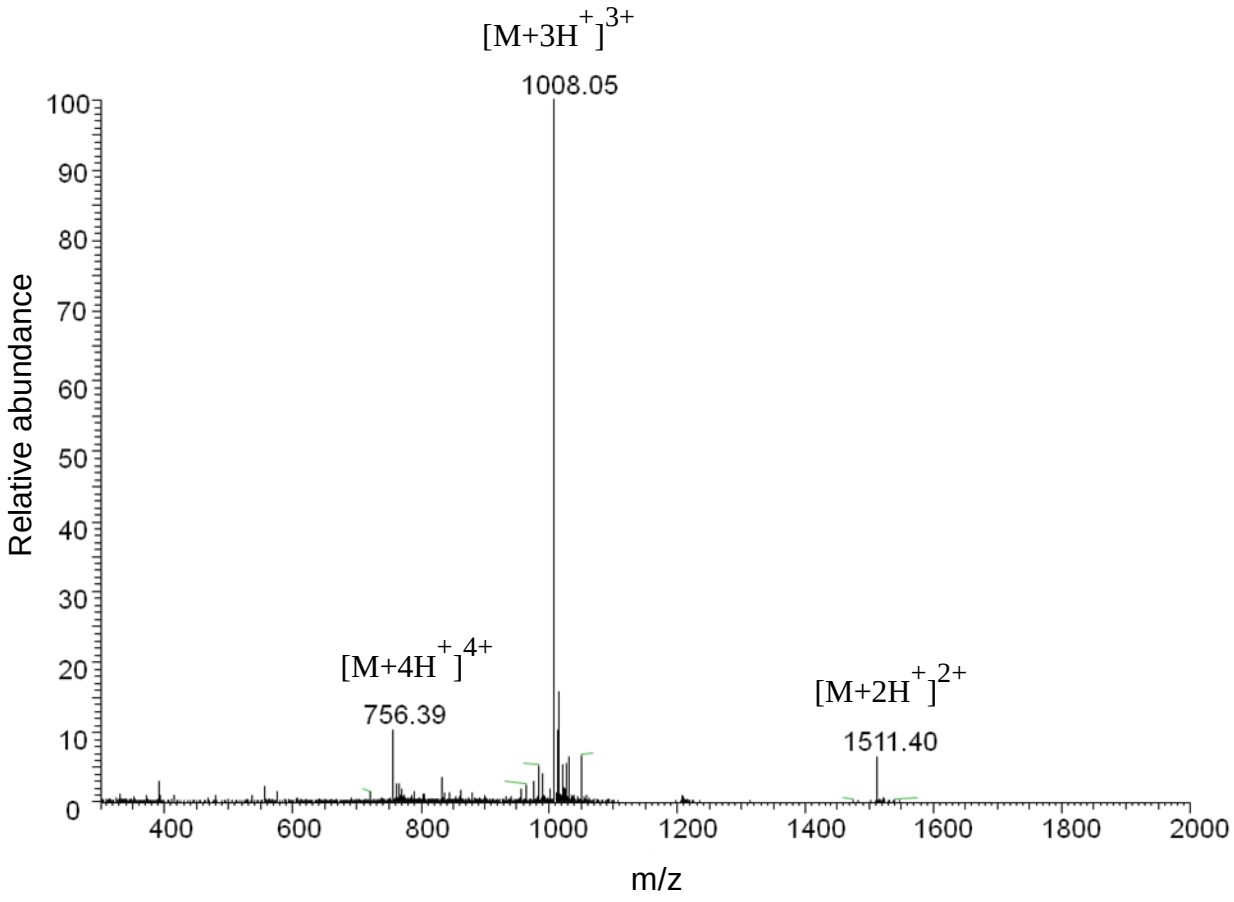

Figure 2.4: Mass spectrum of C-peptide to show the purity of the fraction collected. Spectrum shows the $[M+2H]^{2+}$, $[M+3H]^{3+}$ and $[M+4H]^{4+}$ charged ions.

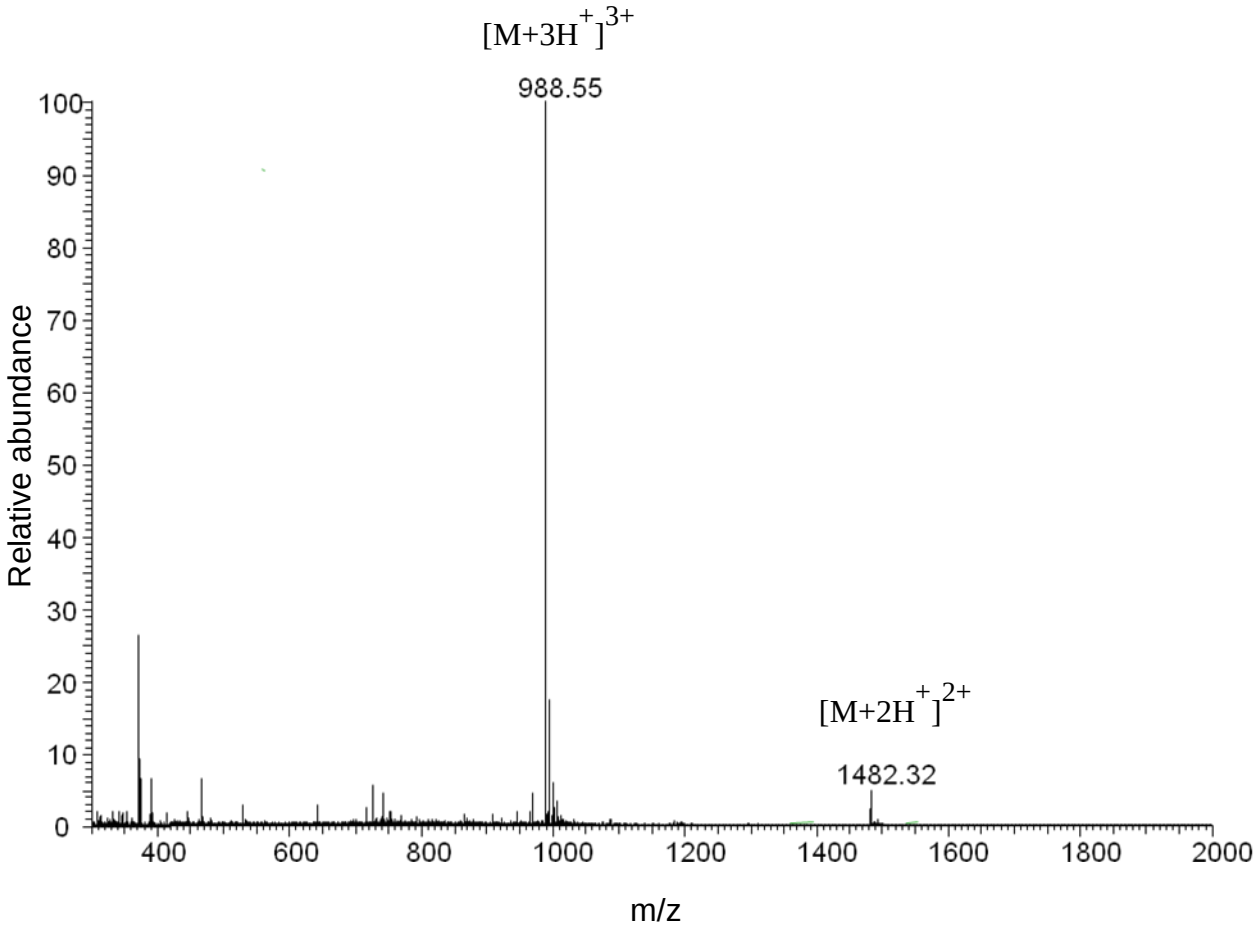

Figure 2.5: Mass spectrum of E27A mutant to show the purity of the fraction collected. The spectrum shows the $[M+2H]^{2+}$, $[M+3H]^{3+}$ and charged ions.

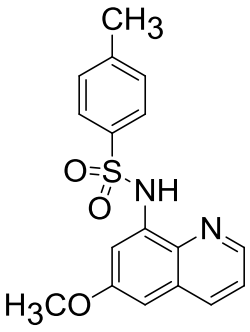

Figure 2.6: Structure of the Zn^{2+} binding probe TSQ

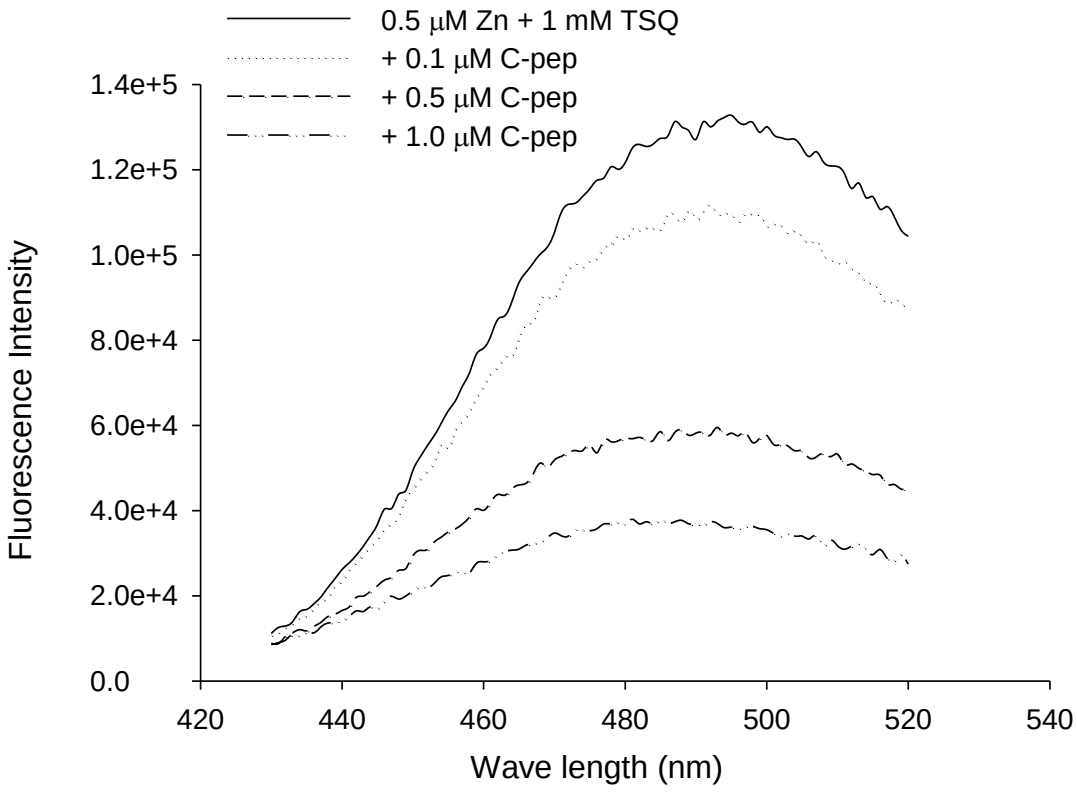

Figure 2.7: Fluorescence intensity of the Zn-TSQ complex with increasing amounts of C-peptide added. The study was performed in 10 mM Tris buffer at pH 7.4.

$$C_{L(b)} / C_{L(f)} = (N - C_{L(b)})\, K \qquad (2)$$

where, r is $C_{L(b)} / C_{P(0)}$ (degree of binding or saturation fraction), $C_{L(b)}$ is concentration of the bound ligand (in this experiment Zn^{2+}), $C_{P(0)}$ is concentration of the binding component (total protein concentration), $C_{L(f)}$ is concentration of the free ligand (Zn^{2+}), n is number of binding sites, N is $nC_{P(0)}$ (binding capacity), and K is intrinsic association constant.[37]

The Scatchard equation follows the assumptions that all ligands are identical (in this experiment Zn^{2+}), the activity will not be altered (except by binding), two independent sets of binding sites are present at maximum, all sites of each set are equivalent, there is no cooperativity, and each set of binding site obeys the mass law action. If there is more than one type of binding site with different binding affinities to the ligand, the Scatchard plot becomes curvilinear. However, during the experiment, this problem was not encountered suggesting the binding sites present at any given time were similar in nature. This may be due to the fact that in the C-peptide molecule, acidic amino acid residues, which are similar in nature, are involved in the binding. The binding parameters can be read from the Scatchard plot. The intrinsic association constant K is equal to the negative value of the slope, and the number of binding sites n or binding capacity N corresponds to the x-axis intercept, depending on whether r or $C_{L(b)}$ was plotted.[37] Here, the concentration of C-peptide was known, so equation (1) was used to represent binding data during the data analysis.

[Zn^{2+}]/ µM	Replicate 1	Replicate 2	Average fluorescence	After background subtraction
0	12360	12360	12360	0
0.1	83520	77880	80700	68340
0.2	182260	198390	190325	177965
0.3	283530	316240	299885	287525
0.4	405340	395620	400480	388120
0.5	521160	514820	517990	505630
0.6	613650	607780	610715	598355

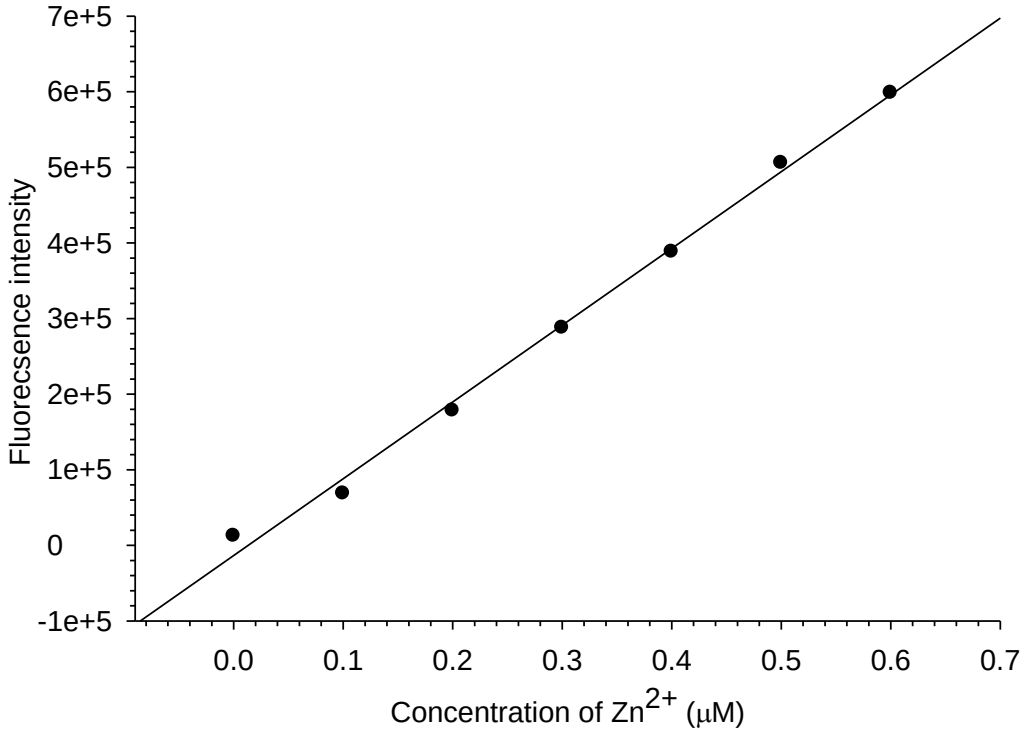

Figure 2.8: Sample calculations for the Scatchard plot analysis of binding data. The Zn^{2+} concentration was changed from 0 to 0.6 µM while keeping the TSQ concentration at 1.5 µM. Data for the standard curve and representation of a standard curve. Simple linear regression yields the equation y = 1000000x – 19131 with a linear fit of R^2 = 0.9973.

$[Zn^{2+}]$/ µM	Average fluorescence	After background subtraction	Free $[Zn^{2+}]$	Bound $[Zn^{2+}]$	r	r / $C_{L(f)}$
0	10360	0				
0.1	40115	29755	0.04753	0.05247	0.52469	11.0387
0.2	109845	99485	0.11533	0.08467	0.84671	7.34167
0.3	187140	176780	0.19048	0.10952	1.09518	5.7495
0.4	269380	259020	0.27044	0.12956	1.29557	4.79053
0.5	363810	353450	0.36226	0.13774	1.37743	3.80237
0.6	466195	455835	0.4618	0.1382	1.38195	2.99251

Table 2.4: Sample calculations for the Scatchard plot analysis of binding data. Data represents the binding system where C-peptide and Zn^{2+} were added in 10 mM Tris buffer at pH 7.4. Zn^{2+} concentration was changed from 0 to 0.6 µM while keeping C-peptide concentration at 0.1 µM and TSQ concentration at 1.5 µM. For each data point, the average fluorescence of two replicate samples was used. The average fluorescence signal was used to calculate the amount of unbound (free) Zn^{2+} concentration, using the standard curve (Figure 2.8). Since the total amount of Zn^{2+} added to the sample was known, the remaining amount of Zn^{2+} was considered to be bound to the C-peptide. The fraction bound (r) and the ratio of fraction bound / free Zn^{2+} concentration (r / $C_{L(f)}$) was calculated. The Scatchard plot was generated by plotting r / $C_{L(f)}$ Vs r.

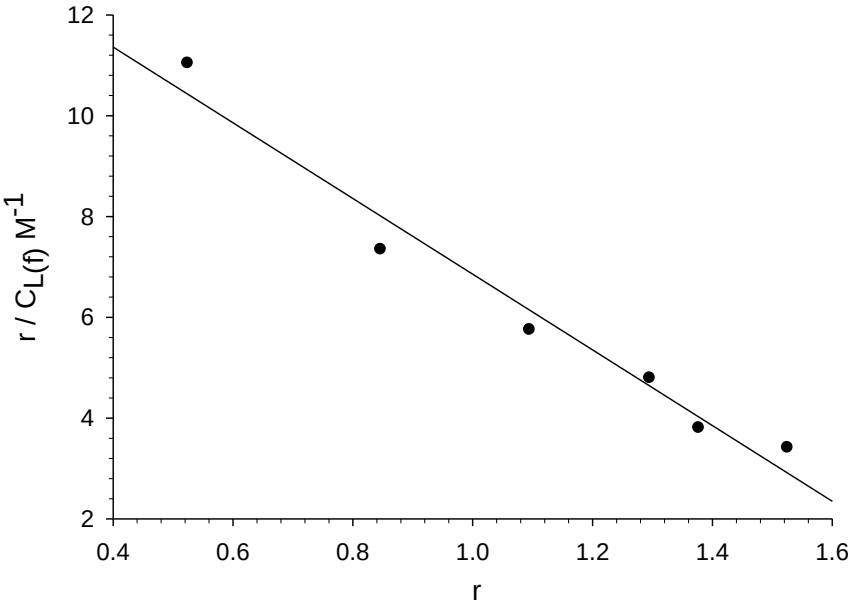

Figure 2.9: Scatchard plot representation of data. The binding system represented is C-peptide and Zn^{2+} in Tris buffer (pH 7.4). Linear regression of data yields the binding constant (K) and number of binding sites (n). For data presented here, K = 7.57 x 10^6 M^{-1} and n = 1.91 (R^2 = 0.9678).

During the course of studies performed, it was discovered that C-peptide must be mixed with Zn^{2+} in water (pH < 6) prior to adding the physiological salt solution (PSS) at pH 7.4. Therefore, in all experiments, C-peptide was allowed to interact with Zn^{2+} in water prior to adding PSS, followed by the cells. If C-peptide was mixed with Zn^{2+} in PSS, the activity was either decreased or absent. During the binding study, C-peptide and Zn^{2+} were mixed both ways to see determine if there is a difference in binding due to the change in pH.

C-peptide has five acidic amino acid residues (Table 2.1) and, including the C-terminal carboxylic group, has a total of six sites that can possibly bind with Zn^{2+}. When mixed in water (pH ~ 5.5) prior to adding the buffer (10 mM Tris at pH 7.4), C-peptide bound to Zn^{2+} with a higher affinity ($K = 1.22 \times 10^{7}$) than when C-peptide and Zn^{2+} were mixed in the buffer ($K = 7.99 \times 10^{6}$) as summarized in Figure 2.10. As shown in Figure 2.11, the number of binding sites per peptide (n) also changed with pH; when Zn^{2+} and C-peptide were mixed at pH 5.5, n was ~1 and at pH 7.4 it increased to ~2. The data suggests that at high pH, the two binding sites with lower binding affinity are available for Zn^{2+}. The higher binding affinity site at pH 5.5 may indicate some kind of chelation of the metal ion by the peptide.

To determine which acidic amino acids have an impact on the Zn^{2+} binding, five single amino acid mutants of C-peptide (Table 2.1) were evaluated for Zn^{2+} binding. In each mutant, one of

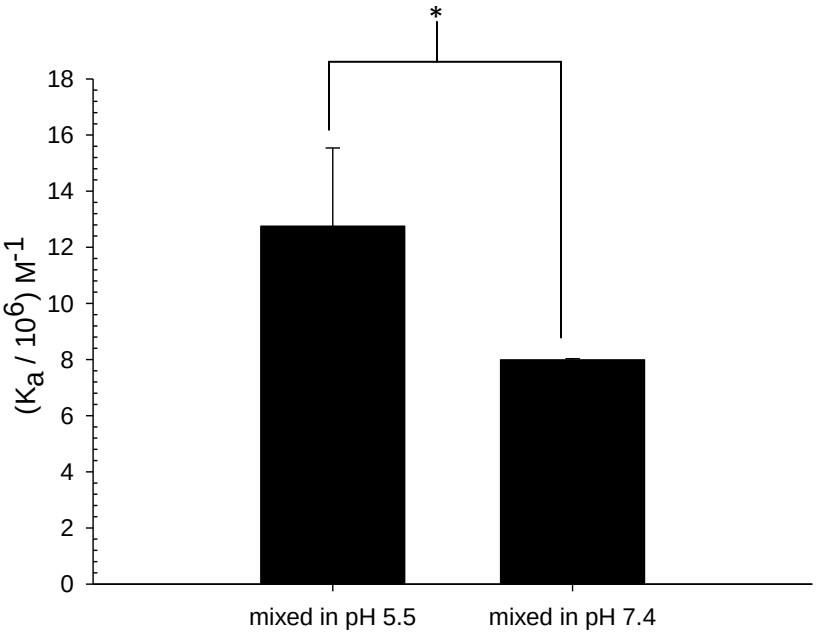

Figure 2.10: The change in the binding constant when Zn^{2+} and C-peptide are either mixed in water (pH~5.5), or in Tris buffer (pH 7.4). Error bars are std. dev. (n = 4), $p^{*} < 0.03$.

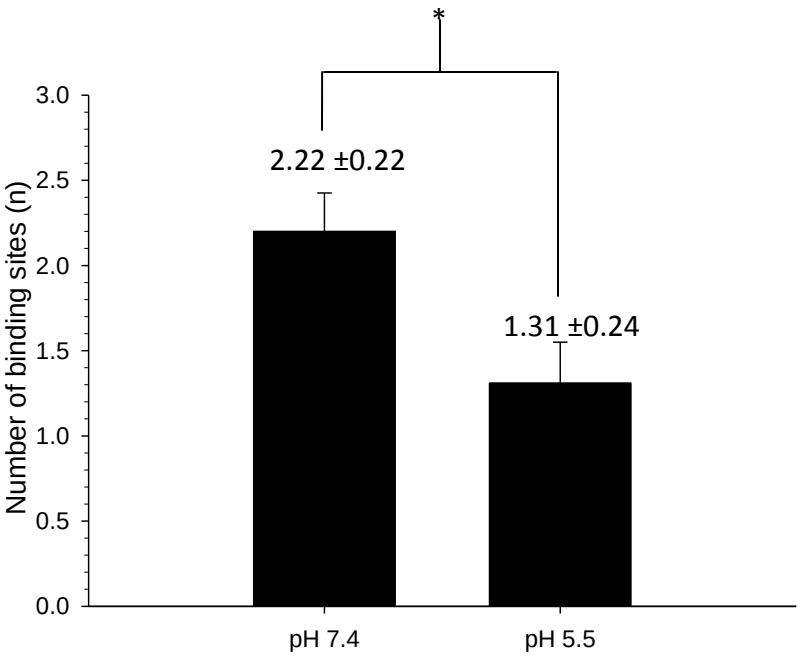

Figure 2.11: The change in the number of binding sites (n) when Zn^{2+} and C-peptide are either mixed in water (pH~5.5), or in Tris buffer (pH 7.4). Error bars are std. dev. (n = 4), $p^{*} < 0.01$.

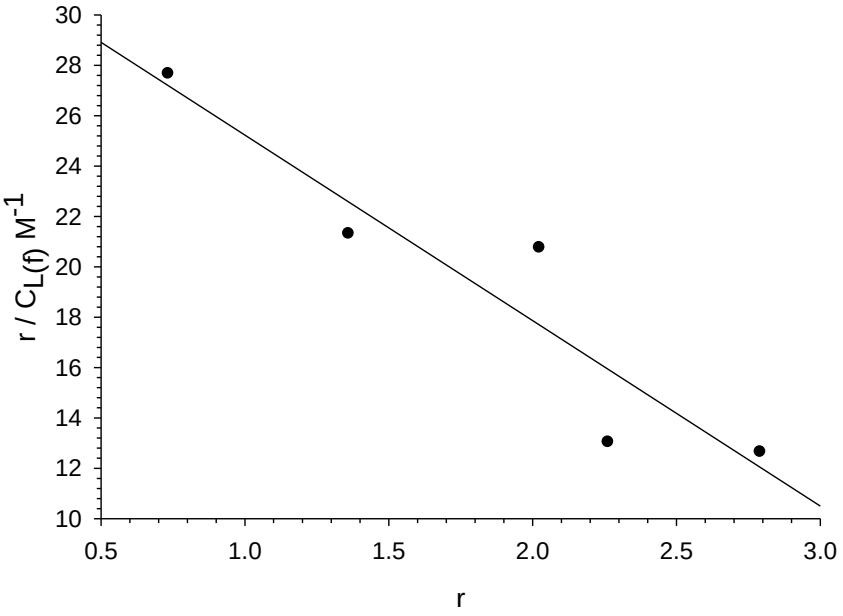

Figure 2.12: Representation of binding data using a Scatchard plot. The binding system represented is E27A and Zn^{2+} mixed in water (pH 5.5) and brought up in Tris buffer (pH 7.4). Linear regression of data yields K = 7.36 x 10^6 and n = 4.42 (R^2 = 0.8747).

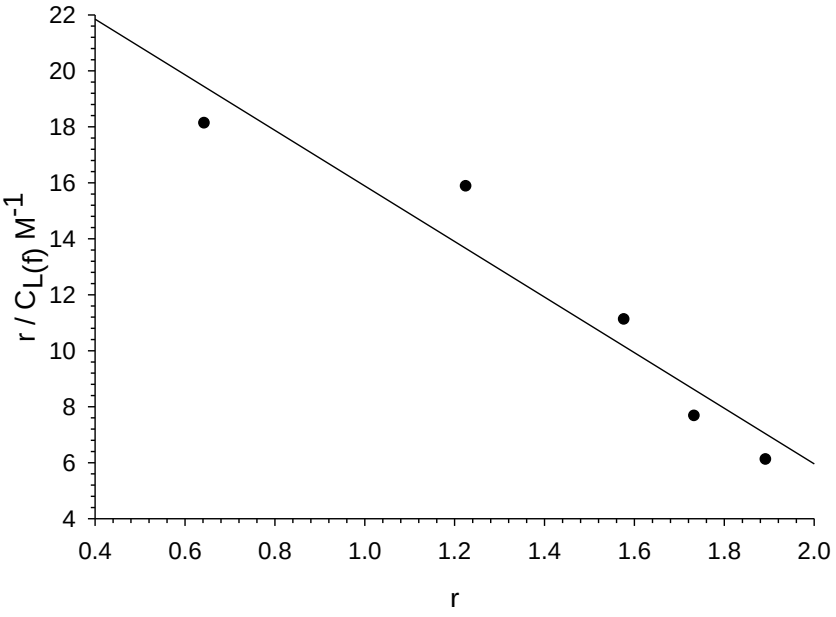

Figure 2.13: Representation of binding data using a Scatchard plot. The binding system represented is D4A and Zn^{2+} mixed in water (pH 5.5) and brought up in Tris buffer (pH 7.4). Linear regression of data yields K = 9.93×10^6 and n = 2.60 (R^2 = 0.9131).

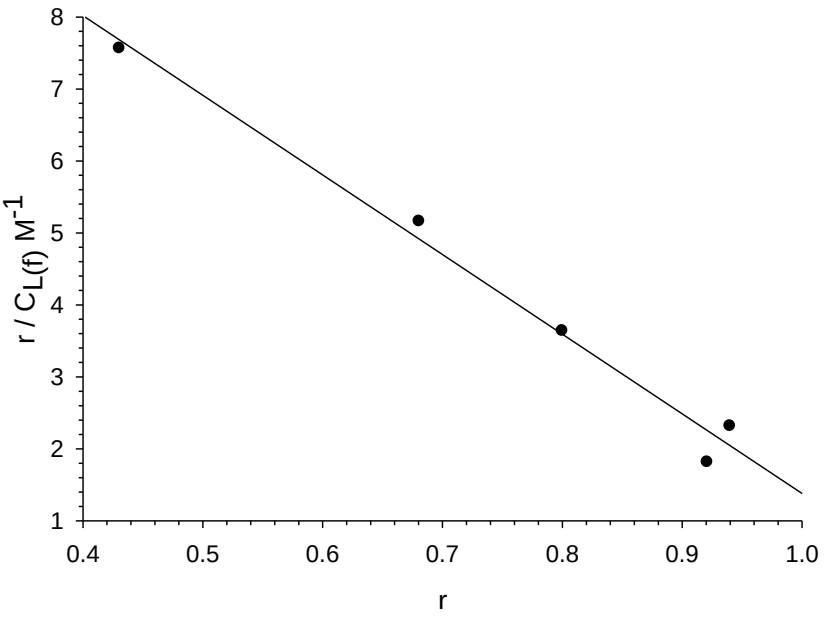

Figure 2.14: Representation of binding data using a Scatchard plot. The binding system represented is D4A and Zn^{2+} mixed in water (pH 5.5) and brought up in Tris buffer (pH 7.4). Linear regression of data yields K = 11.07 x 10^6 and n = 1.12 (R^2 = 0.9842).

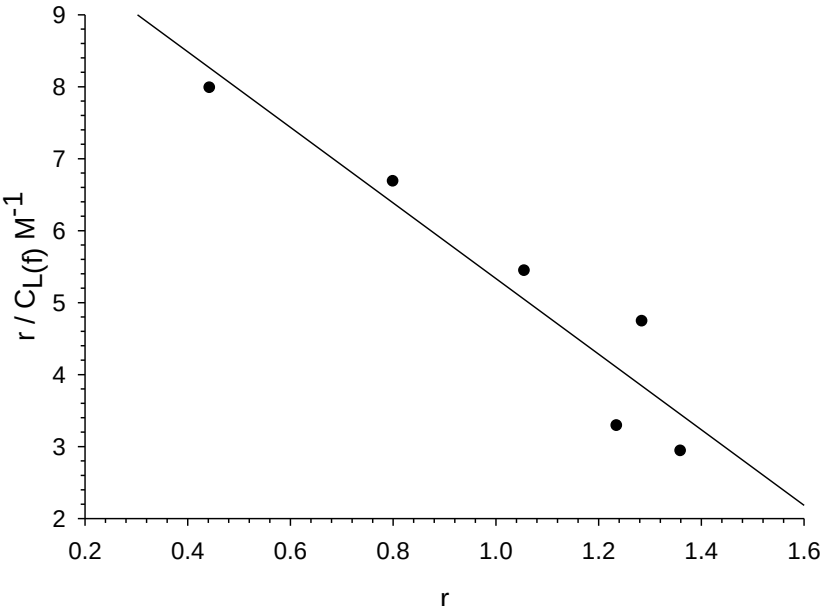

Figure 2.15: Representation of binding data using a Scatchard plot. The binding system represented is E3A and Zn^{2+} mixed in water (pH 5.5) and brought up in Tris buffer (pH 7.4). Linear regression of data yields K = 5.25 x 10^6 and n = 2.01 (R^2 = 0.8921).

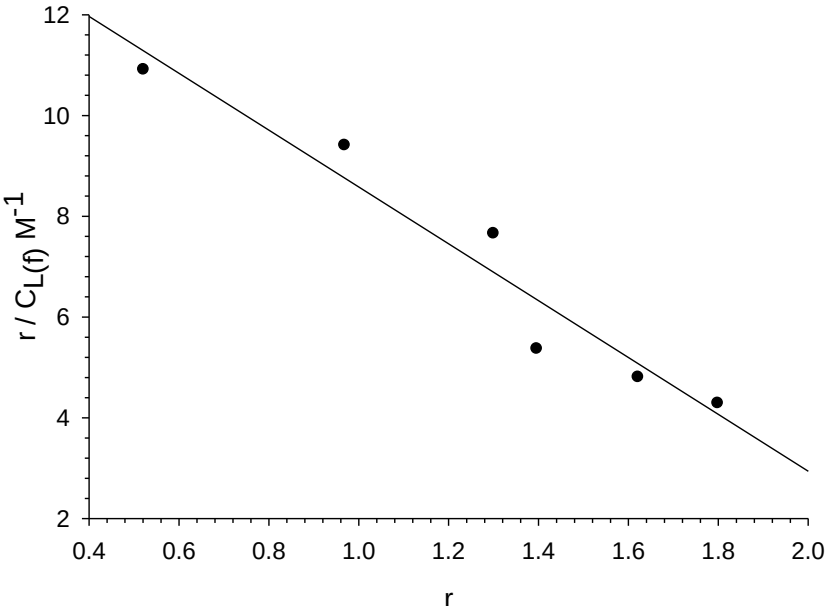

Figure 2.16: Representation of binding data using a Scatchard plot. The binding system represented is E11A and Zn^{2+} mixed in water (pH 5.5) and brought up in Tris buffer (pH 7.4). Linear regression of data yields K = 5.64 x 10^6 and n = 2.42 (R^2 = 0.9388).

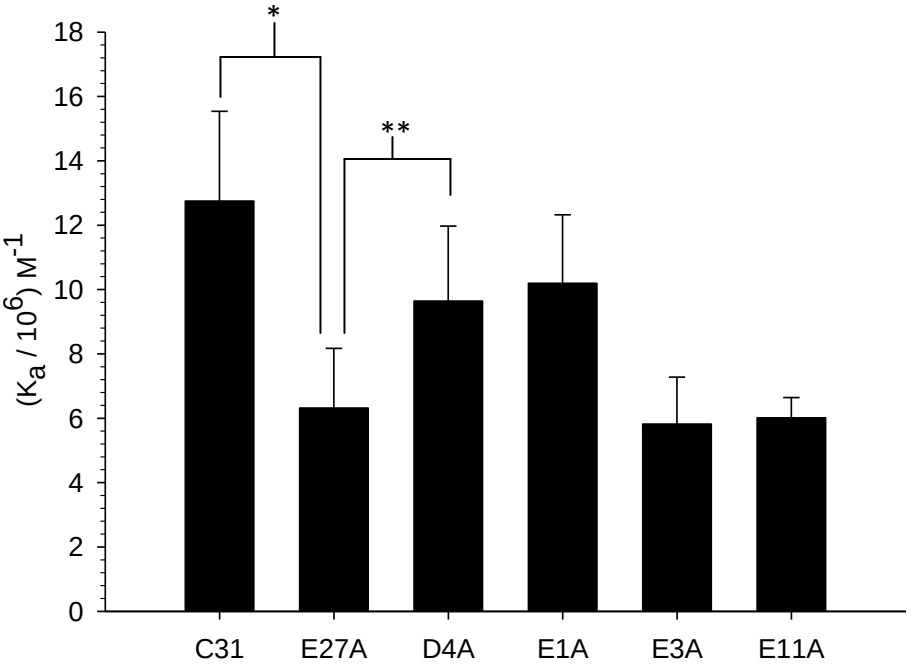

Figure 2.17: The change in the binding constant between Zn^{2+} and C-peptide and its single amino acid mutants. The peptides and Zn^{2+} were mixed in water (pH 5.5) and 10 mM Tris buffer was added (pH 7.4). Wild type peptide (C31) had the highest binding constant while the mutants E27A, E3A and E11A had significantly lower binding constants. Error bars are std. dev. (n = 4), $p^{*} < 0.01$, $p^{**} < 0.03$.

the acidic amino acids was substituted with alanine. Figures 2.12-2.16 show the Scatchard representations for the binding of each mutant with Zn^{2+}. Figure 2.17 shows the change in binding constant for each C-peptide mutant and wild type peptide. C-peptide has the highest binding constant ($K = 1.22 \times 10^7$) while the mutants E27A, E3A and E11A had significantly lower (less than 50%) binding constants. Data shows that the glutamate residues at positions 27, 3 and 11 had the most effect on Zn^{2+} binding, while the aspartate residue at position 4 had only a moderate effect (change in binding constant only by ~ 20%) on the binding. The glutamate residue at position 1 did not have a significant effect on binding.

The number of binding sites on the E27A was the highest at ~4 while the C-peptide had the lowest number at ~1 (Figure 2.18). A higher number of binding sites on the mutant suggests an open structure for the mutant, where Zn^{2+} has access to more binding residues. In comparison, C-peptide having only one site suggests a more closed structure. Other mutants had around ~2 binding sites per peptide possibly indicating a partially open structure. The binding data for C-peptide and its mutants are summarized in Table 2.5.

C-peptide has no observable secondary structure in aqueous solution. The far-UV CD spectrum of C-peptide shows a minimum around 200 nm and an absence of bands in 210-230 nm region (Figure 2.19)**.** This is a signature typical of an unfolded peptide.[38] After the addition of Zn^{2+}, the minimum around 200 nm decreased. This was only observed when the Zn^{2+} concentration was at 1:1 with C-peptide concentration. When higher concentrations of Zn^{2+} were present, the change in the minimum around 200 nm was decreased or absent (Figure 2.20).

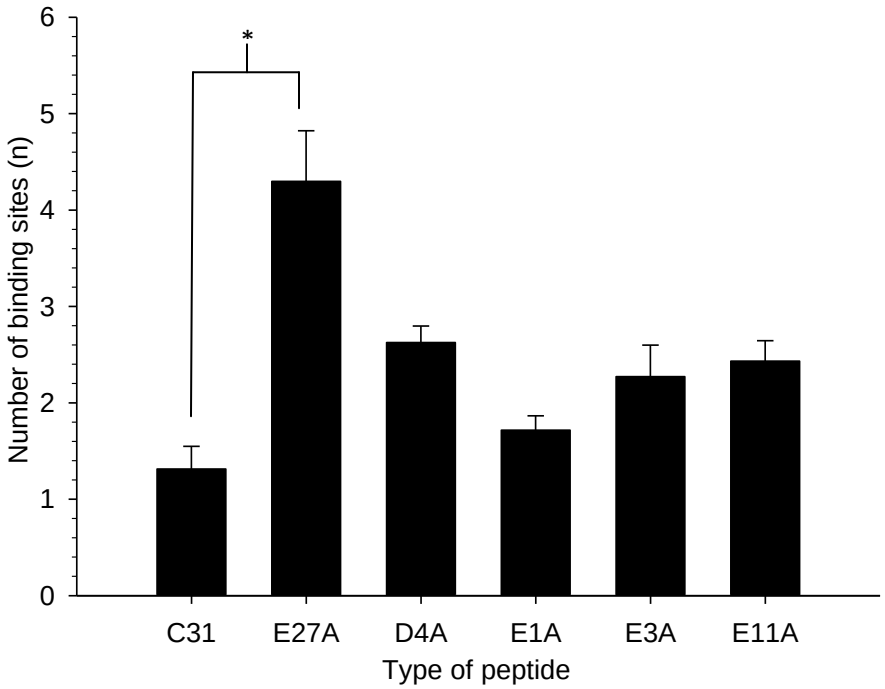

Figure 2.18: The change in the number of binding sites (n) for Zn^{2+} on C-peptide and its single amino acid mutants. The peptides and Zn^{2+} were mixed in water (pH 5.5) and 10 mM Tris buffer was added (pH 7.4). Wild type peptide (C31) had only ~1 binding site per molecule while the mutant E27A had ~4. Other mutant had ~2 sites per molecule. Error bars are STDEV (n = 4), $p^{*} < 0.01$.

Mutant	Binding constant (K) x 10^6 M^{-1}	Number of binding sites (n)
C-peptide	12.24 ± 2.78	1.31 ± 0.24
E27A	6.32 ± 1.85	4.29 ± 0.52
D4A	9.64 ± 2.33	2.62 ± 0.17
E1A	10.19 ± 2.12	1.71 ± 0.15
E3A	5.82 ± 1.46	2.27 ± 0.33
E11A	6.01 ± 0.63	2.43 ± 0.21

Table 2.5: Summary of the change in the binding constant (K) and number of binding sites (n) of C-peptide with each single amino acid mutation. The peptides and Zn^{2+} were mixed in water (pH 5.5) and brought up in 10 mM Tris buffer (pH 7.4). Error is std. dev. (n = 4).

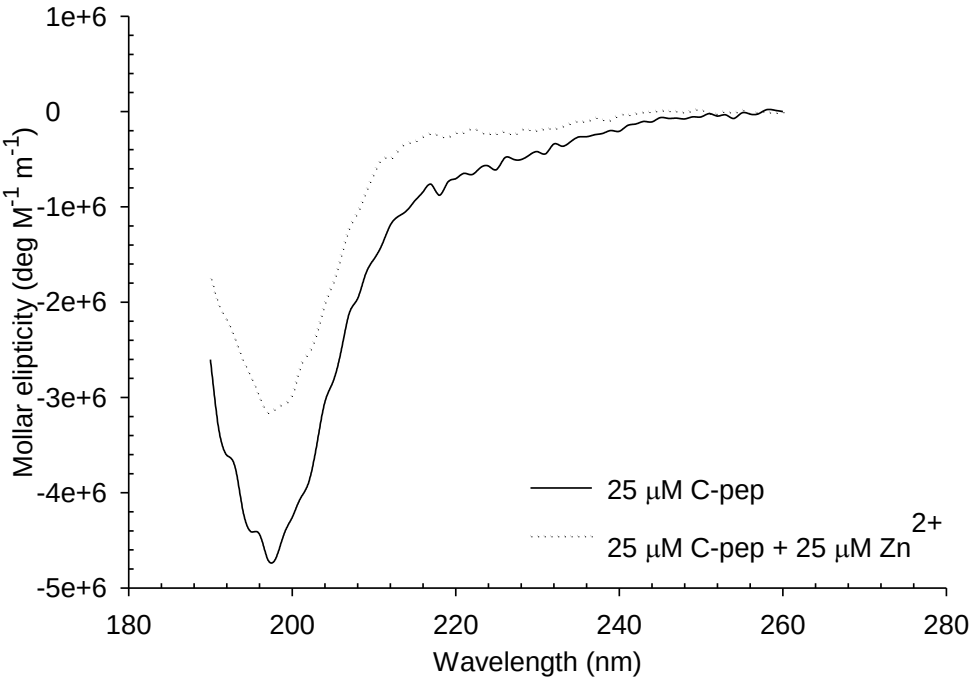

Figure 2.19: The CD spectrum of C-peptide in the far UV region, with and without the addition of Zn^{2+}. In the presence of Zn^{2+}, the minimum around 200 nm decreased. C-peptide and Zn^{2+} were mixed in water (pH 5.5) and Tris buffer (pH 7.4) was added. The spectra were recorded in Tris buffer. The buffer spectrum was subtracted from each spectrum.

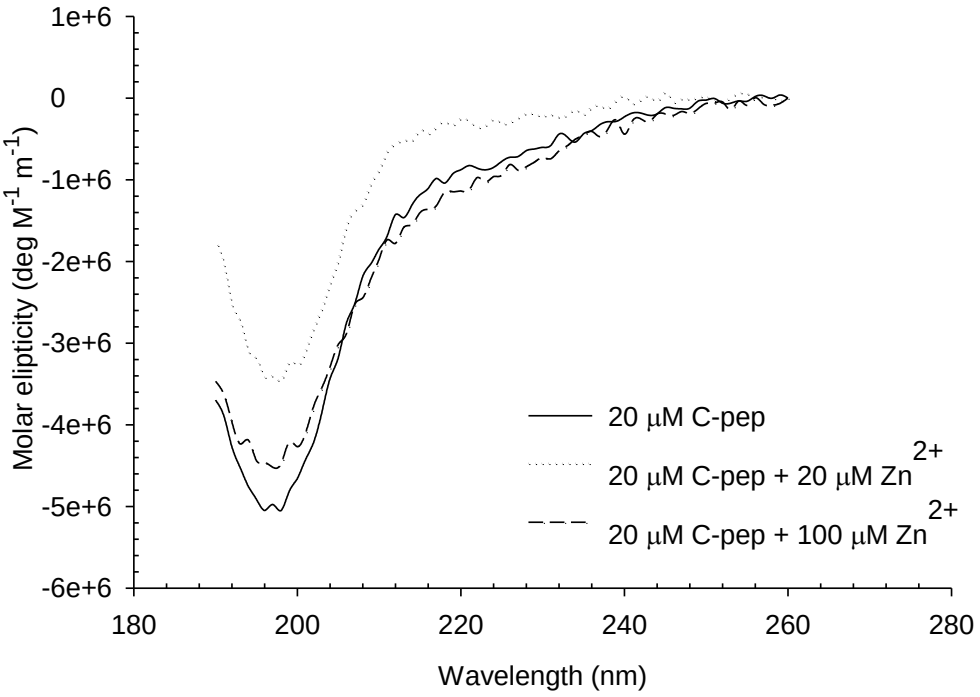

Figure 2.20: The CD spectrum of C-peptide in the far UV region, without addition of Zn^{2+}, at 1:1 Zn^{2+} to C-peptide ratio, and at 1:5 Zn^{2+} to C-peptide ratio. C-peptide and Zn^{2+} were mixed in water (pH 5.5) and Tris buffer (pH 7.4) was added. The spectra were recorded in Tris buffer. The buffer spectrum was subtracted from each spectrum.

This may suggest that any folding present at 1:1 Zn^{2+} to C-peptide disappears when excess Zn^{2+} is added to the sample. Metal ion-induced formation of secondary structure of proteins is reflected by a decrease in the minimum around 200 nm accompanied by an increase in the negative ellipticity around 225 nm.[38-40]

After metal addition to C-peptide, the decrease of the minimum at 200 nm was evident. However, once the solvent peak was subtracted, any changes in 210-230 nm region were not evident and reproducible. This may have been caused by the change of the signal being too low due to the low concentrations of C-peptide used in the studies, keeping in mind that higher concentrations approaching sub mM levels would likely result in aggregation. Collectively, the data suggest that with the addition of Zn^{2+}, C-peptide undergoes some kind of conformational change. This change, however, is very subtle as observed by the CD spectra. It is interesting that 1:1 Zn^{2+} to peptide ratio was optimal for the change, because from the binding studies it is suggested that most possibly in the low pH environment in the pancreatic β-cells, a 1:1 complex of C-peptide and Zn^{2+} is formed.

2.4 Discussion

C-peptide has been shown to ameliorate diabetic complications, including neuropathy, nephropathy, and microvascular complications. However, the structure-function relationship of C-peptide and the molecular mechanism behind its physiological function(s) have not yet been completely elucidated. Unlike insulin, which has 18 conserved amino acid residues,[41] C-peptide

varies among species in terms of amino acid sequence as well as chain length.[42] This variation has long-supported the view that C-peptide does not exert any biological activity *in vivo*.[41, 43, 44]

There are, however, well conserved amino acids among mammalian species: Glu3, Gln6, Glu11, Leu12, Leu26, Glu27 and Gln31, and to some extent Glu1 and Val7.[41] Glu3 and Glu27 are ~70% conserved, while the other amino acids are ~50% conserved among species. Out of these amino acids, Glu1 and Gln31 are present at the proteolytic cleavage sites of proinsulin, and the codons for Gln6 and Val7 line an intron/exon junction in the proinsulin gene,[44] explaining why these amino acids are conserved. The conservation of the other amino acids among species may also indicate their relevance in biological activity.

The conserved glutamate residues, especially Glu27 has been found to be essential for biological activity in many studies. The C-terminal pentapeptide, but not the mid-part of the peptide, was able to displace the wild type C-peptide from membranes,[45] increase intracellular Ca^{2+} concentrations,[46] and stimulate Na+/K+ ATPase activity.[47] In type 1 diabetic patients, the pentapeptide increased red blood cell deformability.[48] The presence of the Glu27 residue has proven to be essential for the phosphorylation of ERK 1/2 by C-peptide. Loss of glutamate residues at positions 3 and 11 also resulted in a decrease in ERK 1/2 phosphorylation. [41] During an *in vivo* study using STZ-induced diabetic rats, the 27-31 fragment (C-terminal pentapeptide) elicited the same increase in glucose utilization as that of the native peptide. The 1-26 fragment did not have any effect.[49] Contradictory to other findings, the C-terminal tetrapeptide fragment 28-31 also showed the same activity as the native peptide during this study.[49] The intrinsic

activity of the tetrapeptide GSLQ has not been well studied. However, during another study it was proven to be unable to displace C-peptide from cell membranes, indicating the necessity of Glu27 for membrane interactions.[50] Fragments 11-15 and 11-19 failed to displace the native C-peptide from membranes[50] or increase glucose utilization in diabetic rats, even though they both contain a glutamate residue at position 1.[49] However, these fragments have shown limited activity in stimulating Na+/K+-ATPase in renal tubular cells.[47]

Considering the above reports, it can be safely concluded that the acidic amino acids, especially the glutamates, are important in conserving the cellular interactions and biological activity of C-peptide. Studies in the Spence group have shown the ability of C-peptide to elicit an increase in ATP release and glucose utilization in some cells found in the circulation: red blood cells, endothelial cells and macrophages.[32, 51] Interestingly, this activity of C-peptide was only observed when C-peptide was able to interact with a transition metal ion such as Cr^{3+}, Fe^{2+} or Zn^{2+}. When C-peptide or a metal alone was added, the increase in ATP release was not observed. If C-peptide requires metal-activation for its function, this may explain why the conserved glutamate residues have a significant effect on C-peptide activity. Keeping this in mind, the interaction between C-peptide and Zn^{2+} was investigated here in order to closely examine their binding.

As reported here, when compared at pH 5.5 and pH 7.4, C-peptide has a higher binding constant of 1.22×10^{7} with Zn^{2+} at pH 5.5. This value is lower than what is reported for insulin in literature ($K_{Zn} \sim 1 \times 10^{9}$).[14, 52] This is understandable because C-peptide binding is through

carboxylate groups, while with insulin, Zn^{2+} is bound in well defined binding sites consisting of histidyl imidazole rings with higher affinity for Zn^{2+}.[52] The higher binding constant of C-peptide to Zn^{2+} at pH 5.5 and n (highest number of ligands bound) being 1.10 ± 0.20 suggest the possibility that more than one residue is involved in binding. However, at physiological pH, n increased to 2.20 ± 0.22 indicating two Zn^{2+} ions binding to the peptide with a lower binding constant. Therefore, as expected, pH has an effect on how Zn^{2+} interacts with C-peptide.

β-cell granules are rich in Zn^{2+} (mM levels), so there is a high chance for C-peptide to bind to Zn^{2+} inside the granule where the pH is ~5.5. This is important because the high binding affinity will allow the C-peptide to keep the metal ion until it reaches the circulation or an interacting cell found in the circulation (such as the RBC). However, comparing the binding affinities of C-peptide and insulin for Zn^{2+}, it is unlikely that Zn^{2+} binds to C-peptide first. More likely Zn^{2+} binds first to the insulin moiety in the proinsulin and remaining Zn^{2+} binds to C-peptide moiety.[25] It is also likely that C-peptide binds to the Zn^{2+} being released when the Zn-insulin hexamer breaks down. This might explain how C-peptide enhances insulin disaggregation, as previously reported.[22] When C-peptide binds and removes free Zn^{2+} from the medium, it may induce more Zn-insulin hexamers to dissociate and release more Zn^{2+}.

Another implication of this phenomenon is that C-peptide might protect β-cells from exposure to unhealthy levels of Zn^{2+}. Uncontrolled amounts of free Zn^{2+} released from granules

can cause β-cell death.[30] In STZ-induced diabetic rats, a mild complexing agent, clioquinol, was able to reduce cell death by removing Zn^{2+} ions.[31] It might be possible that C-peptide serves as an endogenous chelator for free Zn^{2+}, serving a protective role during exocytosis.

The binding of metals such as Cr^{3+} and Ca^{2+} mostly occur at deprotonated carboxylate groups of acidic amino acid residues.[53, 54] The binding of metals in metalloproteins occurs almost exclusively through side chain interactions, via atoms such as O, N and S. Coordination through O and N in the peptide bond is rare in metalloproteins, although carbonyl O and deprotonated amide N are good ligands in smaller molecules. This may be due to steric hindrance for accessibility. Glutamic and aspartate residues are very common in the binding sites and prefer to bind monodentate (through one carboxyl O) rather than in a bidentate fashion. Zn^{2+} is predominantly found tetrahedrally bound to metalloproteins, and being a borderline metal ion (according to hard soft acid base concept) has higher affinity for N and O, usually from neutral ligands. Being a transition metal with filled d shell (d^{10}), the arrangement of the ligands around Zn^{2+} is the result of the equilibrium between the attractive electrostatic metal-ligand forces and the repulsion of the bound residues.[55] Given that C-peptide has no histidine residues, it was assumed that any interaction with C-peptide is via the acidic amino acid residues. To determine the effect from each acidic amino acid residue in C-peptide, single amino acid mutants of C-peptide were examined for Zn^{2+} binding.

From the data reported here, it is clear that when Glu27 is substituted with alanine (mutant E27A), the binding affinity of C-peptide for Zn^{2+} is decreased by ~50% (Figure 2.17), indicating that this glutamate residue is involved in the interaction. Interestingly, the substitutions at Glu3 and Glu11 also had similar effect on the binding affinity, indicating these amino acids are also important for the correct binding of Zn^{2+} to the C-peptide molecule. The substitution of Asp4 and Glu1 by alanine had the least effect on binding. When the number of binding sites per molecule was considered, the E27A mutant had the highest number of sites per molecule (~4), while the wild type C-peptide and the E1A mutant had the lowest number of sites (~1). All of the other mutants had intermediate numbers of binding sites. The number of binding sites, combined with the binding affinity data, suggests that the Glu1 residue is least important during binding of Zn^{2+}. Furthermore, the higher number of binding sites may indicate an open structure where more binding residues are available for Zn^{2+}, while a lower number of binding sites may indicate the structure is closed or more hindered. Thus, in comparison, the wild type C-peptide would have a more closed structure while E27A would have a more open structure.

Keltner *et. al.*, the first studies performed to investigate the binding between C-peptide mutants and Zn^{2+}, used ESI-MS to study the affinity of these mutants to Zn^{2+}.[56] They observed that multiple residues were responsible for Zn^{2+} binding in addition to Glu27 in the wild type C-peptide. There was lack of Zn^{2+} binding in the C-terminal pentapeptide region of the E27A mutant, indicating that the Zn^{2+} binding in this region may need the Glu27 residue. According to the abundance of the Zn^{2+}-bound precursor ions in the mass spectrum, the mutants had lower

affinity for Zn^{2+} than the wild type peptide. Further investigation with the synthesized C-terminal pentapeptide EGSLQ and its mutant AGSLQ, showed significantly less Zn^{2+} binding to pentapeptides compared to the full length peptide. Between the two pentapeptides, AGSLQ had a lower binding constant than EGSLQ, agreeing with results from the full length peptide. However, while EGSLQ showed preferential Zn^{2+} binding at the N-terminus, AGSLQ also showed Zn^{2+} binding that localized at the C-terminal, indicating that in the absence of the Glu1, either the terminal carboxylate, hydroxyl group of Ser3 can participate in non-specific Zn^{2+} binding. All these observations agree with the experimental results discussed in this chapter.

No secondary structure has been observed for C-peptide under physiological conditions,[57] although the N-terminal region has shown the potential to form an α-helix in trifluoroethanol (TFE).[41, 57] If a helical structure is formed during C-peptide interactions, the conserved Glu3 and Glu11 will be located on the same side of the helix[41] indicating they can participate together during an interaction. However, any metal ion influence on the C-peptide secondary structure has not yet been reported. The CD spectra of C-peptide shows a minimum around 210 nm, which is typical of a random structure, and the addition of metal in a 1:1 ratio showed a change in this minimum. However, the change observed was not enough to confirm that C-peptide could form a specific structure such as an α-helix in the presence of the metal. It is more likely that the metal binding decreased the total randomness of the peptide by coordinating to more than one residue. Whether this change is important for physiological activity has not yet been determined. However, the fact that higher Zn:C-peptide ratios seem ineffective in either

decreasing the peak around 200 nm or increasing ^{65}Zn interactions with the red blood cells (Figure 2.21) seems to indicate that there is a connection between the structure and activity. Most probably, the chelation in 1:1 complex decreases the total randomness of the peptide structure.

Previous studies by the Spence group have shown the necessity of the metal ion for C-peptide activity in increasing glucose utilization and ATP release of red blood cells and endothelial cells.[32, 51] In the study by Keltner *et. al*., the single amino acid C-peptide mutants (Table 2.1) showed lower effectiveness in increasing red blood cell ATP release, while the C-terminal pentapeptide EGSLQ showed similar effectiveness as that of the wild type peptide.[56] E27A and the mutant pentapeptide AGSLQ showed the lowest effectiveness, again indicating the importance of Glu27 for bioactivity. Interestingly, the activity of the mutant E1A was not significantly different from that of the wild type ($p = 0.025$),[56] agreeing with the binding data presented in this chapter showing that the mutation at position 1 had least effect on the binding of the peptide with Zn^{2+}.

Recent unpublished data in the Spence group using radioactive ^{65}Zn, performed by Suzanne Letourneau, indicates that C-peptide is needed for Zn^{2+} uptake to the red cells at nM concentrations of Zn^{2+}(Figure 2.21). Interestingly, E27A could not elicit this effect on the Zn^{2+} uptake, showing that Glu27 was necessary for this activity. Further investigation using different Zn^{2+}:C-peptide ratios show that the 1:1 Zn^{2+}-C-peptide complex is most efficient in delivering Zn^{2+} to the red cells. However, this phenomenon was observed only when serum albumin was

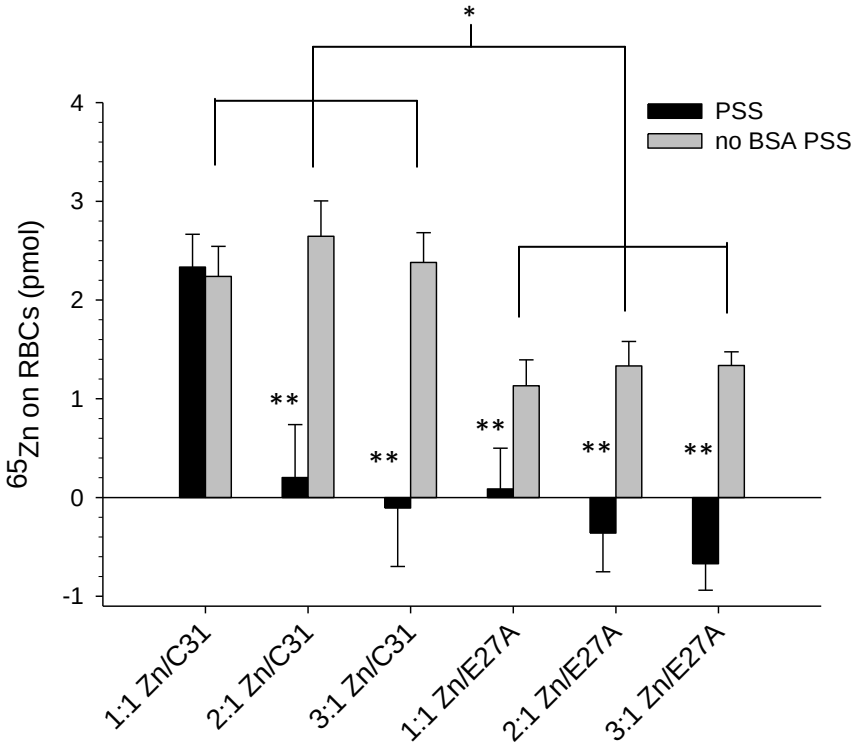

Figure 2.21: $^{65}Zn^{2+}$ uptake in red blood cells stimulated by either wild type C-peptide (C31) or E27A mutant. E27A is less effective in stimulating $^{65}Zn^{2+}$ uptake, compared to wild type C-peptide. The 1:1 ratio of C-peptide to $^{65}Zn^{2+}$ is most effective for $^{65}Zn^{2+}$ uptake in the presence of serum albumin (in PSS). Error bars are SEM (n =3), $p^{*} < 0.05$, $p^{**} < 0.001$ compared to 1:1 Zn to C-peptide in PSS.

present. When albumin was absent from medium, even complexes prepared at higher Zn^{2+}:C-peptide ratios were found capable of delivering the Zn^{2+} to the red cells. A suitable explanation for this is that serum albumin, which has a comparably high binding affinity for Zn^{2+} ($K = 9.54 \times 10^7$) can remove the loosely bound Zn^{2+} from an open C-peptide chain. However, the high affinity 1:1 Zn-C-peptide complex was able to retain the Zn^{2+}. Notably, even in the absence of albumin, E27A did not show the full effect of the wild type peptide, showing the necessity of Glu27 for Zn^{2+} binding and probably for cellular interaction. Combined data from binding studies and ^{65}Zn uptake studies suggests that C-peptide can serve as a carrier for Zn^{2+}.There is a complex relationship between Zn^{2+} and glucose homeostasis. It is well documented that Zn^{2+} plays a role in insulin synthesis, storage and release from β-cells.[58] Experimentally created zinc deficiency in rats decreased glucose tolerance by reducing insulin secretion and sensitivity.[59] Zinc deficiency and hyperzincuria are frequently observed in the pathogenesis of diabetes mellitus.[28]

Apart from the effect on insulin synthesis and secretion, the ability of Zn^{2+} itself to increase glucose metabolism has been reported. The ability of zinc to exert insulin-like effect,[60, 61] and the ability of Zn(II) salts with low toxicity to have anti-diabetic effect in diabetic experimental mice have been reported.[62, 63] In healthy human subjects, acute oral zinc supplementation improved glucose disposal in response to an intravenous glucose tolerance test,

via an increase in non-insulin-dependent glucose uptake. No change in insulin secretion or sensitivity was observed.[64]

Impaired glucose tolerance and diabetes are features of cirrhosis.[65] Interestingly, poor zinc status is also common in this disease.[66] In a study with patients having cirrhosis, after zinc supplementation, glucose disposal was improved, and it was entirely due to non-insulin mediated glucose uptake.[67] The way in which zinc increases glucose disposal is still speculative. Because the glucose metabolizing enzymes are not metalloenzymes, the effect of zinc was suggested not to be at this level. Therefore, the effect of zinc was suggested to be exerted via the glucose transporters in the cell membranes through enhanced glucose transporter activity, or by modification of the transporter.[67] Enhanced glucose effectiveness may not only mean increased activity of GLUT1 and GLUT2 (insulin independent glucose transporters), but also activation of GLUT4 (insulin dependent glucose transporter) via a mechanism distinct from the insulin related pathway.[68] In rat adipocytes, Zn^{2+} induced glucose transport by increasing the number of glucose transporters in the cell membrane. However, the mechanism did not involve the insulin receptor kinase activity, indicating a post receptor mechanism for the metal action.[69]

Another important effect of Zn^{2+} is stabilizing the cell membrane structure.[70] Zinc deficiency increased the osmotic fragility of rat erythrocytes.[71, 72] It also significantly decreased the concentration of zinc in the rat erythrocyte plasma membrane and altered the phospholipid composition, especially the cholesterol to phospholipid ratio. Zinc repletion restored the zinc levels in the plasma membrane, but did not rapidly restore the phospholipid composition. The

protein profile of the membrane also remained the same. Considering that zinc status rapidly changes red cell membrane pathology, but not its composition, suggests that the role of zinc in cell membrane integrity may be more subtle.[73] Therefore, attention was also given to reversible processes occurring at the cell membrane or cytoskeleton and that can be affected by zinc status. These include: maintenance of sulfhydryl groups in reduced state,[74] activation of receptors and ion channels,[75] and alteration of membrane bound enzyme activities.[76, 77]

As mentioned previously, there is evidence that zinc can alter cholesterol to phospholipid ratios in cell membrane. The amount of cholesterol in the membrane changes the cell membrane fluidity, increasing cholesterol levels making the membrane less fluid.[78, 79] The rigidity of the cell membrane can decrease deformation induced ATP release from cells.[80, 81] Thus zinc can have an indirect effect on ATP release from cells. Interestingly, erythrocytes of multiple sclerosis (MS) patients, who have higher amounts of zinc [82, 83] and lower amounts of cholesterol[84] in their red blood cell membrane compared to healthy controls, release increased amounts of ATP when subjected to deformation.[85]

This evidence suggests that Zn^{2+} can have direct and indirect effects on cellular metabolism and ATP release of red blood cells. The molecular mechanism behind the stimulation of red blood cell derived ATP release by C-peptide is still not fully understood. However, the fact that C-peptide appears to require Zn^{2+} to stimulate ATP release, combined with its ability to increase Zn^{2+} uptake in red blood cells, suggest the possibility of the

involvement of Zn^{2+} in the mechanism. This aspect will be further discussed in chapter 3 of this dissertation.

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Chapter 3 - Interaction of Zn^{2+}-activated C-peptide with the Red Blood Cell and the Consequent Change in the Red Blood Cell Derived ATP Release

3.1 Glucose Metabolism of the Red Blood Cell

The red blood cell is the most common cell type found in the blood tissue with a density of approximately 5 million cells/µL of whole blood. It is a biconcave disk shaped cell with an average disk diameter of 6-8 µm, which enables the cell to travel through small capillaries.[1] Red blood cells carry oxygen, bound to hemoglobin, to tissues and organs. These cells are produced through erythropoiesis in the bone marrow and are sequestered by the spleen or liver at the end of their life span of ~120 days.[2] An average human red blood cell completes one circulation through the body in about 20 seconds. During maturation, the cells become devoid of a nucleus and other organelles such as mitochondria, Golgi apparatus and endoplasmic reticulum (ER). Due to the lack of a nucleus, red blood cells do not have the ability to synthesize novel proteins, and have limited capability to repair cellular damages.[3]

As red blood cells lack the necessary organelle for oxidative phosphorylation of glucose (mitochondria), the energy requirement of these cells is fulfilled through glycolysis and the consequent anaerobic conversion of pyruvate to lactate (Figure 3.1). Glucose enters the red blood cell mainly through GLUT1, which is an insulin-independent facilitative glucose transporter. Red blood cells depend constantly on extracellular glucose for survival because they do not store glycogen. Almost 95% of the glucose entering the red blood cell is subjected to glycolysis in the cytosol of the cell, producing the energy molecule ATP. ATP is used by the red blood cell for various metabolic processes and to maintain cellular shape and deformability.[4]

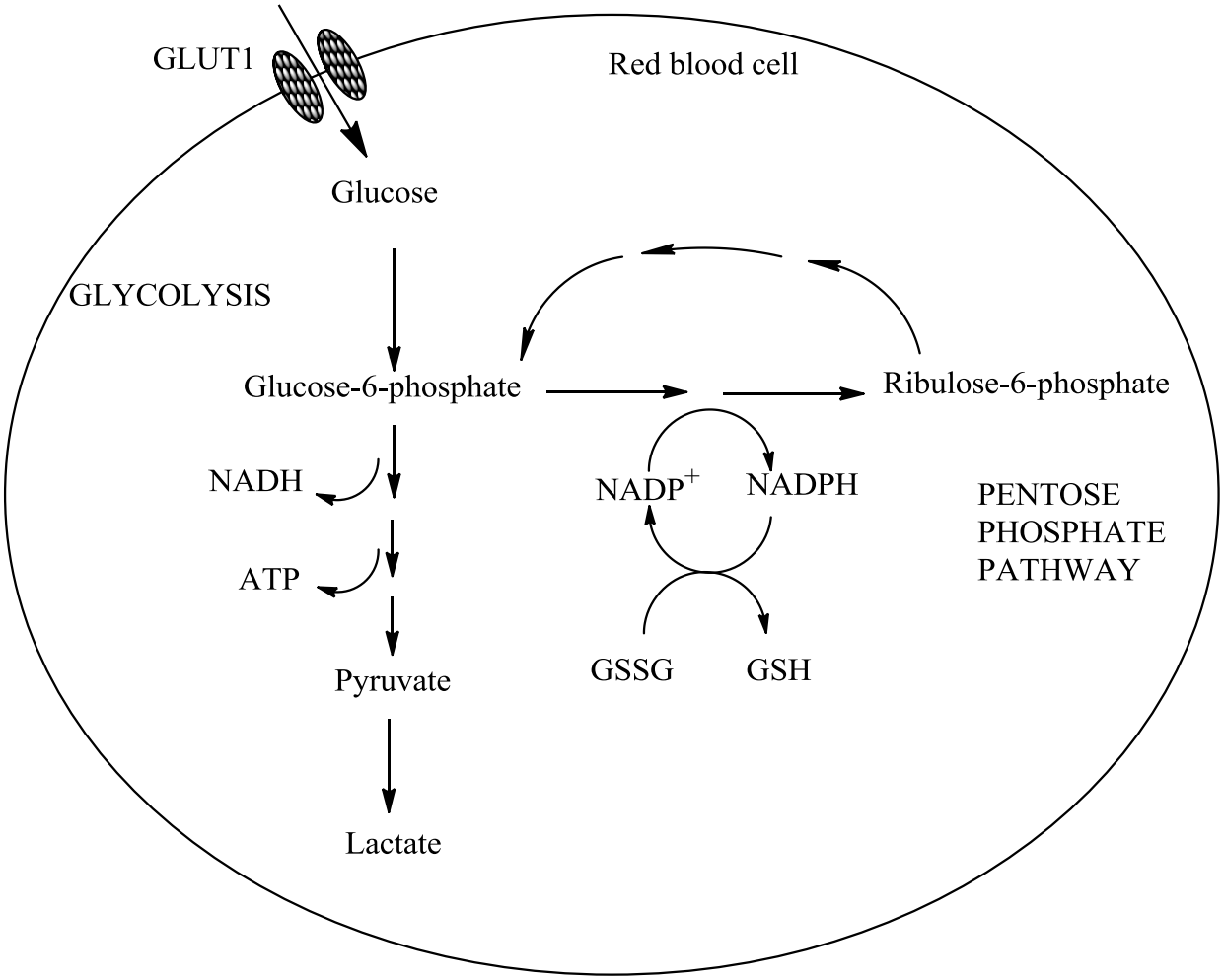

Figure 3.1: Glucose metabolism in the red blood cell under physiological glucose concentrations. The majority of the glucose entering the cell undergoes glycolysis, providing ATP for cellular needs. The remainder enters the pentose phosphate pathway, generating NADPH. NADPH in turn keeps glutathione in its reduced form (GSH), which protects the red blood cell from oxidative damage.

For example, ATP is used to drive the Na^+/K^+-ATPase pump, which is largely responsible for maintaining the red blood cell volume and membrane potential.[5]

Along with ATP, the reduced form of nicotinamide dinucleotide (NADH) is also produced during glycolysis. NADH is needed to convert the oxidized form of hemoglobin, methemoglobin, back to hemoglobin, with the aid of the enzyme methemoglobin reductase. NADH is a co-factor for this enzyme.[6]

At physiological conditions, 3-11% of the glucose utilized by a human red blood cell goes through the pentose phosphate pathway (PPP).[7] The reduced form of nicotinamide adinine dinucleotide phosphate (NADPH) is generated exclusively through the PPP. NADPH is necessary to keep the glutathione in the red blood cell in a reduced state. Glutathione, in turn, reduces the oxidative stress of the red blood cell, and helps to maintain the cell membrane deformability.[8] Oxidative stress in the cell is increased by the presence of reactive oxygen intermediates, and glutathione relieves this stress by participating in the cellular defense system against oxidative stress by reducing disulphide linkage of proteins and other cellular molecules, or by scavenging free radicals and reactive oxygen intermediates.[9]

3.1.1 Red Blood Cell Derived ATP Release and Its Effect on the Blood Flow

As described previously, red blood cells generate ATP via anaerobic glycolysis, and they contain mM amounts of ATP.[10] Red blood cells can release ATP to the extracellular space in response to changes in the local environment.[11] Lowering of pH,[12] hypoxia,[13] and change in

osmotic pressure,[14] are examples of such situations. Mechanical deformation can also induce the red blood cells to release ATP.[15-17] ATP is a charged molecule, therefore it is believed to cross the cell membrane through ion channels, termed ATP binding cassettes.[18, 19] The cystic fibrosis transmembrane conductance regulator (CFTR) is such a channel aiding the movement of ATP out of the red blood cell.[15]

Red blood cell-derived ATP can stimulate endothelial nitric oxide synthase (eNOS), via binding to P_{2y}-type purinergic receptors on endothelial cells. Nitric oxide (NO) produced by endothelial cells can participate in the relaxation of vascular smooth muscle cells.[20] Thus, red blood cells, under conditions of limited perfusion, can release ATP to can serve as a determinant of the local blood flow.[21] Impaired ATP release from red blood cells is associated with many diseases, such as cystic fibrosis,[15] pulmonary hypertension,[22] and diabetes.[23, 24]

In diabetes, lowered endothelial NO production,[25, 26] increased NO degradation,[27, 28] and increased platelet and leukocyte adhesion to the endothelium have been shown to lead to the vascular complications of the disease. Red blood cells of diabetic patients are also less deformable than those of healthy humans,[29] mostly due to decreased NADPH levels, and although they contain increased amounts of intracellular ATP than non-diabetic red cells, release reduced amounts of ATP in response to stimuli such as osmotic stress and mechanical deformation.[8, 23] Therefore, it is believed that abnormal ATP release from red blood cells contributes to the vascular disease in diabetic patients.[23]

C-peptide has shown the ability to ameliorate vascular complications in diabetic patients, by increasing endothelial NO production,[30] red blood cell deformability[31] and red blood cell Na^+/K^+-ATPase activity.[30, 32] C-peptide can increase the ATP release from red blood cells from both human and animal controls, but only in the presence of a metal ion such as Cr^{3+}, Fe^{2+} or Zn^{2+}.[33-35] The implications of this ATP release in healthy and diabetic patients and the role of the metal ion is further investigated in this chapter.

3.2 Experimental

3.2.1 Preparation of Reagents

Purified water with 18.2 MΩ resistance (Easypure® II ultrapure water system, Barnstead) was used for all experiments to ensure absence of metal ion contamination. All reagents were prepared and stored in polypropylene tubes whenever possible, and any glassware used was acid-washed prior to use to avoid any metal-ion contamination.

C-peptide (Genscript, Piscataway, NJ), purified using RP-HPLC and purity verified by ESI-MS as described in chapter 2, was used during experiments. 0.25 mg of C-peptide was dissolved in 10 mL of water to give a stock solution with final concentration of 8.3 µM. Zn^{2+} stock solution was prepared by dissolving 5.5 mg of zinc (II) chloride (Jade Scientific, Canton, MI) in 500 mL of purified water to obtain a final concentration of 80 µM.

A luciferin/luciferase solution for chemiluminescence assay was prepared by dissolving 2.0 mg of D-luciferin (Sigma Aldrich, St.Louis, MO) in 5 mL of water, and adding the resultant solution into one 50 mg vial of firefly extract (Sigma Aldrich, St.Louis, MO).

Zinc-containing insulin (Zn-Ins) and Zinc-free insulin (Ins) were purchased from Sigma Aldrich (St. Louis, MO). The Zn^{2+} content of the Zn-Ins and Ins was analyzed using the Zn^{2+} fluorescent probe TSQ (N-(6-Methoxy-8-quinolyl)-p-toluenesulfonamide). However, this value might be an underestimation of the Zn^{2+} content as some of the Zn^{2+} might still be bound to insulin. The stock solution of Zn-Ins was prepared by dissolving 1.0 mg of Zn-Ins in 1.00 mL of water to give a final concentration of 0.174 mM. 13.9 µL of this solution was diluted to 5.00 mL in water to give a working solution with a Zn^{2+} concentration of 102 nM. Ins stock solution was prepared by dissolving 4.9 mg of Ins in 5.00 mL of water to give a final concentration of 0.174 mM. 13.9 µL of this solution was diluted to 5.00 mL in water to prepare the working solution with similar concentration of insulin as that of in Zn-Ins working solution.

EDTA stock solution was made by dissolving 0.0187 g of Ethylenediaminetetraacetic acid disodium salt (Fisher Scientific, Fair Lawn, NJ) in 50 mL water to give a final concentration of 1 mM.

3.2.2 Collection and Purification of Red Blood Cells

Whole blood was obtained from either healthy rabbits or humans. White New Zealand male rabbits (2.0-2.5 kg) were anesthetized with ketamine (8 mL/kg, i.m.) and xylazine (1 mg/kg, i.m.) followed by pentobarbital sodium (15 mg/kg, iv). A cannula was placed in the trachea, and the animals were ventilated with room air. A catheter was then placed into a carotid artery for administration of heparin and for phlebotomy. After administration of heparin (500 units, i.v.), animals were exsanguinated and the whole blood collected in a 50 mL tube. Human blood was obtained by venipuncture at the antecubital fossa, and collected into heparinized tubes. Red blood cells were obtained on the day of use.

Blood was centrifuged at 500 *g* at 4 °C for 10 min. The plasma and buffy coat were discarded. Red blood cells were resuspended and washed three times in a physiological salt solution [PSS; in mM, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 12 $MgSO_4$, 21.0 tris(hydroxymethyl)aminomethane, 5.5 dextrose with 5% bovine serum albumin (final pH 7.4)]. The hematocrit was determined using a hematocrit measurement device (CritSpin®, Iris sample processing, Westwood, MA).

3.2.3 Enzyme Linked Immunosorbant Assay (ELISA) to Determine the Amount of C-peptide Interacting with the Red Blood Cell

To study the interaction between C-peptide and the red blood cells, 7% RBC samples were incubated with various concentrations of Zn^{2+}-activated C-peptide. Samples were prepared by mixing the appropriate volumes of 49 µL of Zn^{2+} and 60 µL of C-peptide in 1.5 mL centrifuge tubes, followed by the addition of 793 µL of PSS to avoid lysis of cells, and immediately after that, 100 µL of 70% red blood cells. After mixing, the samples were incubated for 2 hours at 37 ºC, the samples were centrifuged (500 *g*, 3 min) and the remaining C-peptide in the supernatant was determined with an Enzyme-Linked Immunosorbant Assay (ELISA) (Millipore, Billerica, MA) (Figure 3.2). The C-peptide standards were treated similarly to the samples, except that they did not contain red blood cells. To avoid any matrix effects, the standards were made from the supernatant of a 7% RBC sample. The difference between the amount of C-peptide added to the cells and the amount recovered in the supernatant was calculated at each concentration with the aid of a calibration curve. To ensure the differences were not due to C-peptide trapped between cells, another set of samples containing Zn^{2+}-activated C-peptide and red blood cells were immediately centrifuged (500 *g*, 3 min) without

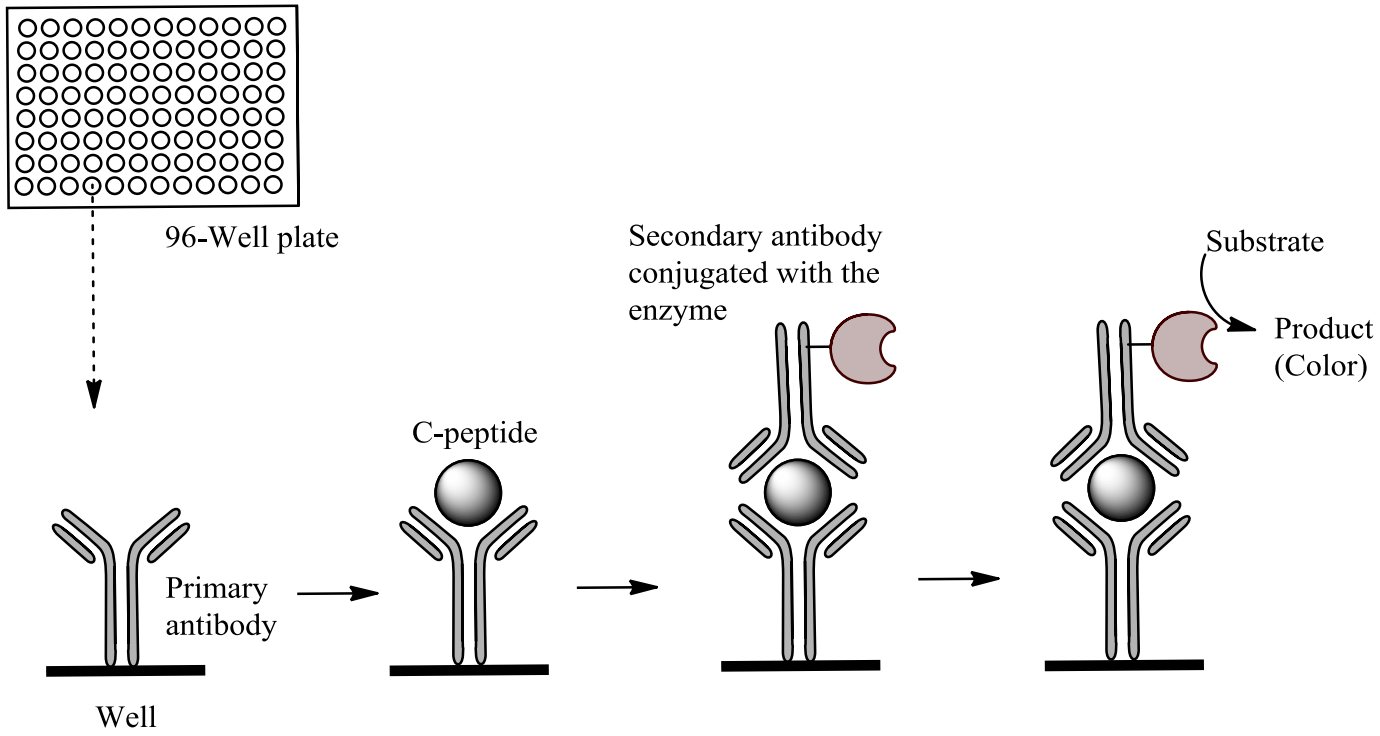

Figure 3.2: Reaction scheme for the detection of C-peptide using the ELISA. The sample containing C-peptide is added into a well in the ELISA plate, where C-peptide binds with the primary antibody coated in the surface of the well. The enzyme conjugated t the secondary antibody converts the added substrate into a colored product. The color change produced is proportional to the amount of C-peptide present in the initial sample.

incubation and the amount of the C-peptide remaining in the supernatant was calculated. To ensure that the missing amount was not due to proteolytic cleavage of C-peptide, aprotinin (a protease enzyme inhibitor, EMD BioSciences, Gibbstown, NJ) was added at 500 KIU / mL to control samples during incubation of C-peptide with red blood cells (KIU = kallikrein inhibition units).

To examine the effect of Zn^{2+} on the interaction of C-peptide with red blood cells, C-peptide at 20 nM concentration, with or without Zn^{2+}, was incubated with red blood cells. Again, the amount of C-peptide remaining in the supernatant after incubation was determined with the ELISA with the aid of a calibration curve.

To investigate the effect of high glucose on the interaction of C-peptide with the red blood cells, the cells were pre-incubated in high glucose PSS (10 mM or 20 mM glucose) for 2 hrs at 37 ºC. At the end of the 2 hrs, 2 nmol of Zn^{2+}-activated C-peptide in PSS was added to 1 mL samples. The final hematocrit was 7%. The samples were incubated at 37 ºC for an additional 2 hrs. Determination by ELISA was carried out as described above to measure the remaining C-peptide in the supernatant.

To measure the amount of C-peptide on the red blood cells directly, after incubation with Zn^{2+}-activated C-peptide, cells were centrifuged at 500 *g* and washed two times in PSS. The red blood cells (100 µL) were lysed in purified water, and the lysate was used as sample for the ELISA to determine the amount of C-peptide present.

3.2.4 Chemiluminescence-based Assay for Determination of ATP Release from Red Blood Cells

To investigate the ability of C-peptide to interact with Zn^{2+} in the presence of insulin, 600 µL of C-peptide was mixed with 490 µL of either Zn-Ins, Ins or Zn^{2+} in water in 15 mL polypropylene tubes. In controls containing EDTA, EDTA was first added to Ins-Zn. After mixing, the solution containing Zn-Ins and EDTA was added to C-peptide. 3410 µL of physiological salt solution (PSS) was added to each sample, followed immediately by the addition of 500 µL of 70% red blood cells to give a final hematocrit of 7%. The final concentration of C-peptide and Zn^{2+} (directly as Zn^{2+} or from Ins-Zn) and EDTA was 10 nM in the solutions. The samples were incubated for 4 hrs at room temperature and the amount of ATP released by the red blood cells in each sample was detected by adding a 100 µL aliquot of luciferin/luciferase mixture to a 200 µL aliquot of the sample and measuring the chemiluminescence (Figure 3.3) after 15 seconds of reaction time with a PMT.

The C-terminal pentapeptide of C-peptide EGSLQ, and its single amino acid peptide AGSLQ, were obtained from Zach Keltner, Reid lab, Michigan State University. The stock solutions of both EGSLQ and AGSLQ were 8.3 µM. Working solution was made by diluting 100.00 µL of the stock solution in 10.00 mL of water to give a final concentration of 83 nM. 600 µL of the pentapeptide EGSLQ / AGSLQ was mixed with 490 µL of either Zn^{2+} or Zn-Ins in water in a manner similar to the experiment with C-peptide described above. 3410 µL of PSS was added, followed immediately by 100 µL of 70% red blood cells. The final concentrations of Zn^{2+} and pentapeptide were 10 nM, and the hematocrit was 7%. After 4 hrs of incubation at

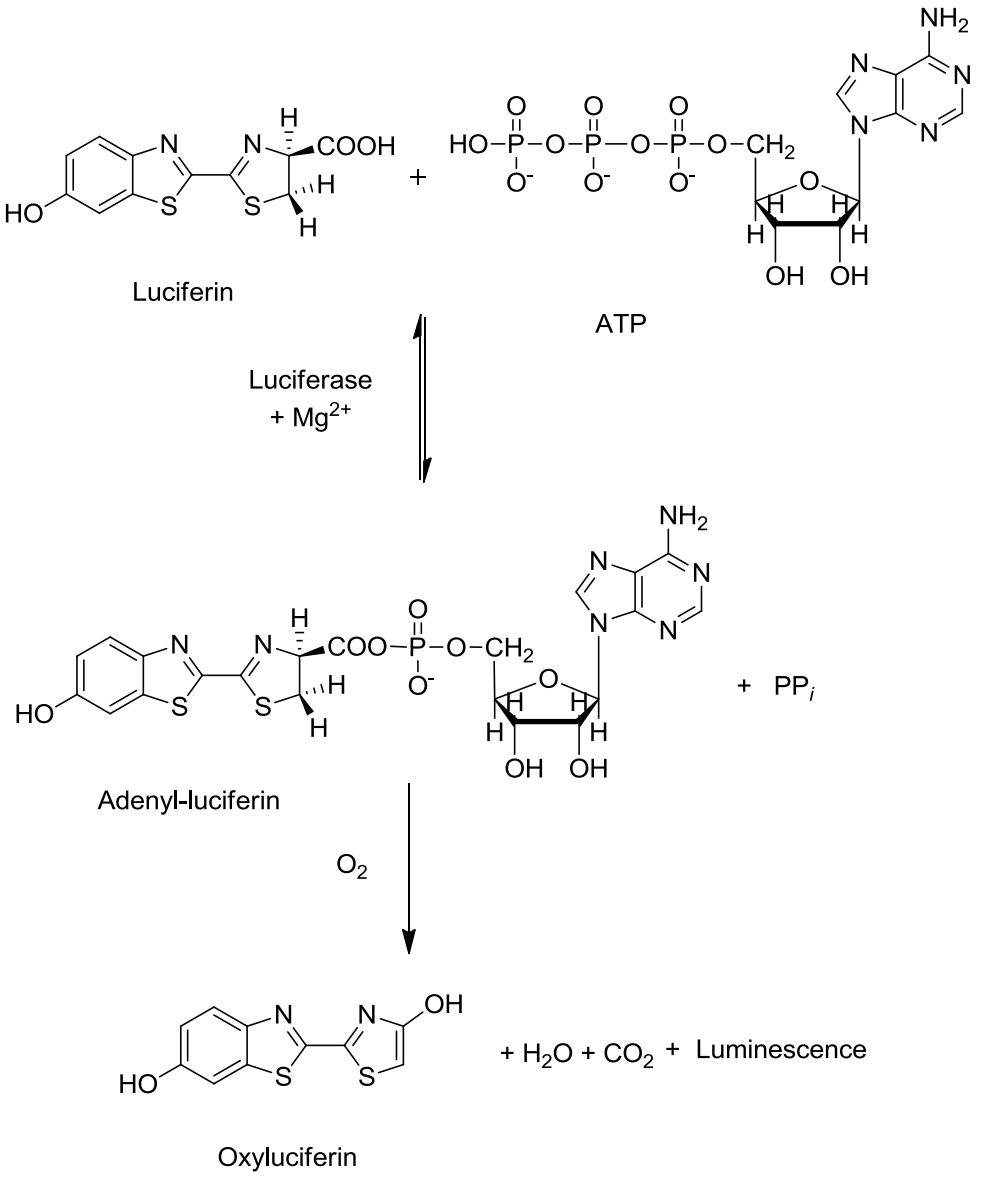

Figure 3.3: Reaction scheme for the detection of ATP using the luciferin/luciferase assay. The chemiluminescence produced is proportional to the amount of ATP present in the sample.

room temperature, the amount of ATP released was measured using the luciferin/luciferase chemiluminescence assay as previously described.

To study the effect of PSS on the Zn^{2+}/C-peptide interaction, Zn^{2+} and C-peptide, freshly prepared in water, were mixed together in 15 mL polypropylene centrifuge tubes (600 µL of C-peptide and 490 µL of Zn^{2+}). 3410 µL of PSS was added to each tube and the solution was left to stand at room temperature for 5, 10, 30 and 60 minutes. After the appropriate time had elapsed, 500 µL of 70% red blood cells were added to each solution to give a final hematocrit of 7%. The final concentrations of Zn^{2+} and C-peptide were 10 nM each. The samples were incubated for 4 additional hours at room temperature and the amount of ATP in each sample was measured with the luciferin/luciferase method as described above.

In order to study the effect of high glucose on the red blood cell-derived ATP release, 500 µL of 70% red blood cells were incubated in 4500 µL of PSS containing 15 mM glucose at room temperature for 6 hrs. After incubation, the cells were centrifuged at 500 *g* for 3 minutes and the 4000 µL of the supernatant removed. 4000 µL of Zn^{2+} activated C-peptide in 15 mM glucose PSS was added to give a final hematocrit of 7% and a final concentration of 10 nM for both Zn^{2+} and C-peptide. The samples were further incubated at room temperature for 3 hrs and the amount of ATP in each sample was detected using the luciferin/luciferase chemiluminescence reaction. A control experiment was carried out using 5.5 mM glucose PSS instead of 15 mM glucose PSS. To determine the effect of Zn^{2+} to C-peptide ratio on the ATP

release, the experiment was carried out at 10, 30, and 50 nM Zn^{2+} concentrations, while keeping C-peptide concentration at 10 nM.

3.3 Results

C-peptide has the ability to increase ATP release from red blood cells in the presence of a metal ion such as Cr^{3+}, Fe^{2+} and Zn^{2+}.[33] Although the ability of C-peptide to bind to cells has been demonstrated,[36, 37] a specific C-peptide receptor has not yet been purified or identified.[38] However, an interaction between the peptide and the red blood cells is expected to exist in order to evoke an increase in the ATP release from the cells. To further investigate this possible interaction, red blood cells were incubated with Zn^{2+} activated C-peptide, and the amount of C-peptide binding to the cells was measured with the aid of an ELISA kit.

C-peptide binding to red blood cells as a function of added C-peptide is shown in Figure 3.4. The amount of C-peptide binding to red blood cells reaches a plateau around 10 pmol of C-peptide added to the cells (1 mL of 7% hematocrit sample with 10 nM concentration of C-peptide), indicating saturation of the red cells with the peptide. This seems reasonable considering that the concentration of C-peptide in the blood stream is in the single-digit nanomolar range. At saturation, the amount of C-peptide bound to the red blood cell was around 2 pmol. Considering the amount of RBCs present in the sample, this gives ~1800 C-peptide molecules binding per red blood cell.

However, Zn^{2+} did not seem to have any significant effect on this interaction (Figure 3.5). When Zn^{2+}-activated C-peptide and C-peptide alone were incubated with red blood cells,

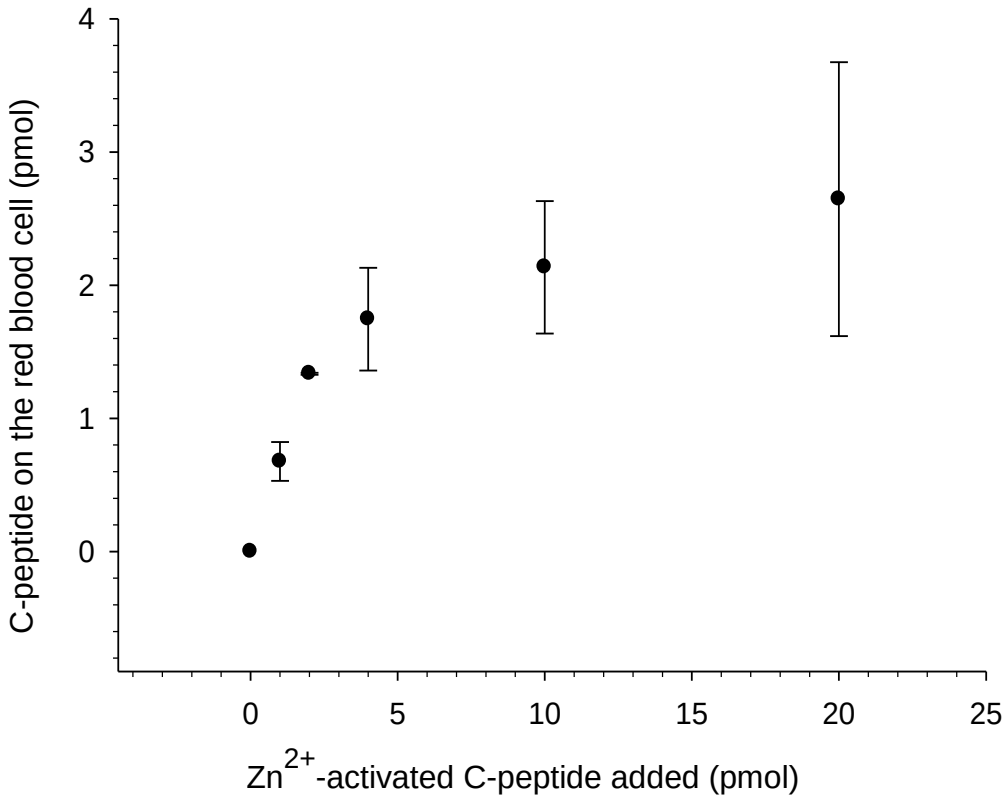

Figure 3.4: The amount of C-peptide binding to red blood cells as a function of C-peptide added. The binding becomes saturated at approximately 10 pmol of C-peptide (10 nM) added to 1 mL of 7% red blood cells. At > 10 pmol of C-peptide added, the curve indicates non-specific binding of C-peptide to the cells. At saturation, about 2 pmol of C-peptide is bound to the cells, giving ~1800 C-peptide molecules per red blood cell. Error bars are ± std. dev. (n = 4).

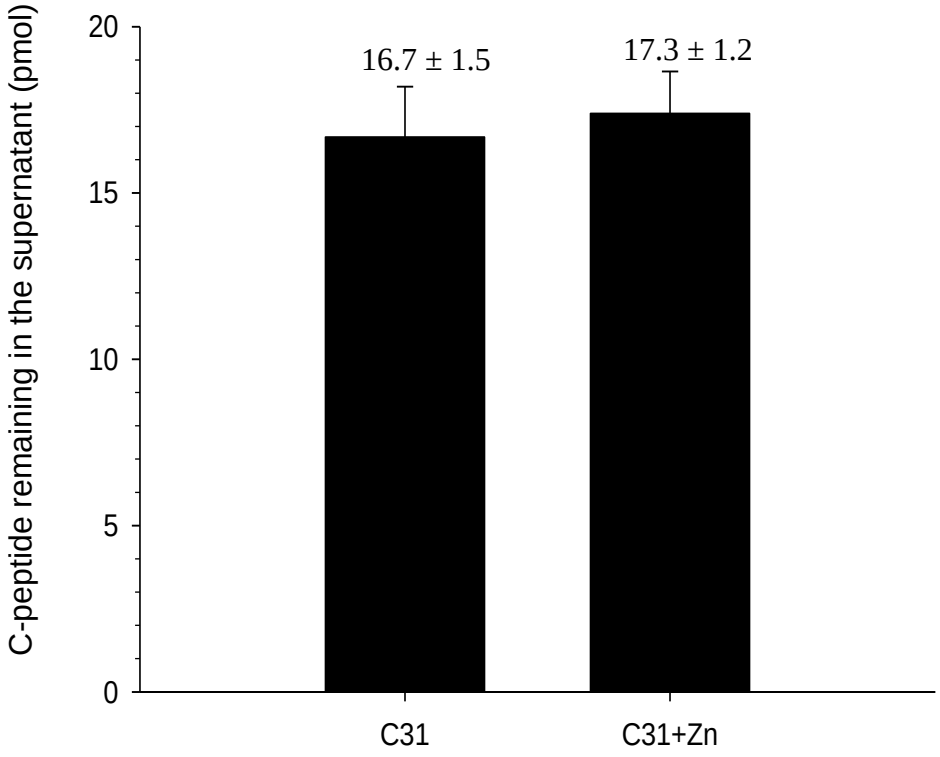

Figure 3.5: The amount of C-peptide binding to red blood cells in the presence and absence of 20 pmol of Zn^{2+} (20 nM). The total amount of C-peptide added to each sample was 20 pmol (20 nM). There was no significant difference in binding of C-peptide to the red blood cells between the two samples ($p > 0.05$). Error bars are ± std. dev. (n = 8).

there was no significant difference between the binding of C-peptide to the red blood cells in the two sample suggesting that C-peptide interactions with healthy red blood cells does not require a metal ion. However, according to previous observations, C-peptide can stimulate red blood cell derived ATP release only in the presence of a metal ion.[33] Therefore it may be possible that C-peptide acts as a delivery mechanism to get the metal ion into the cell, and then the ion itself initiates the metabolic change in the cell.

When red blood cells were incubated with Zn^{2+} activated C-peptide, the ATP release was increased by 64 ± 12% compared to untreated red blood cells (Figure 3.6). When Zn^{2+} containing insulin (Zn-Ins) was added as the Zn^{2+} source instead of $ZnCl_2$, the ATP release increased by 44 ± 11%, showing the ability of C-peptide to become activated from Zn^{2+} supplied from Zn-Ins. When insulin without Zn^{2+} (Ins) was added, the ATP release was increased only by 16 ± 9% indicating the necessity of Zn^{2+} for the proper function of C-peptide. However, when Ins was analyzed for Zn^{2+}, a trace amount of Zn^{2+} (<0.4%) was found. This was 50% lower than the amount of Zn^{2+} found in Zn-Ins. The increase of ATP release of ~16% in the sample with added Ins and C-peptide may be due to this small amount of Zn^{2+} present in the solution.

The notion of Zn^{2+} bound to C-peptide for cellular activity was further strengthened by the addition of EDTA to Zn-Ins prior to mixing with C-peptide. EDTA is a strong metal chelator and can sequester Zn^{2+} from the medium. In this sample, the increase in ATP release was completely reduced to that of the untreated red blood cells (Figure 3.6).

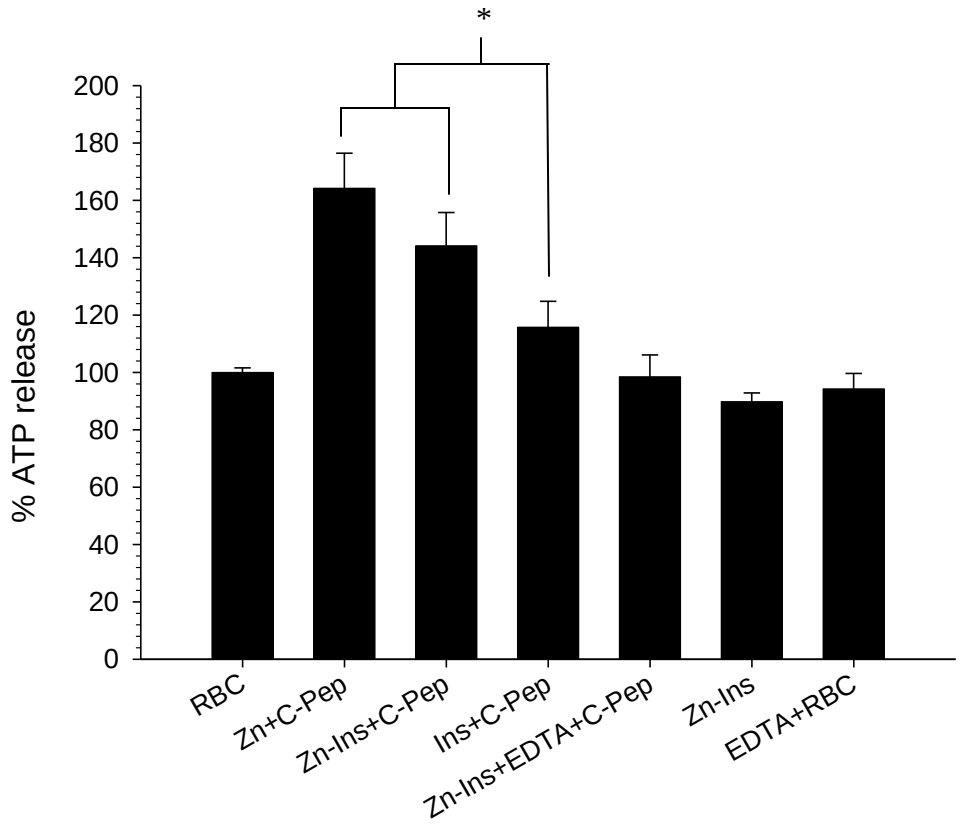

Figure 3.6: The normalized ATP release from red blood cells. When red blood cells were incubated with Zn^{2+} activated C-peptide, the ATP release increased by 64 ± 12% compared to untreated red blood cells. When Zn^{2+} containing insulin (Zn-Ins) was added as the Zn^{2+} source instead of $ZnCl_2$, the ATP release increased by 44 ± 11%. When insulin without Zn^{2+} (Ins) was added the ATP release increased only by 16 ± 9%. Addition of EDTA abolished the ATP release to that of untreated red blood cells. Error bars are ± SEM (n = 4), $p^{*} < 0.05$.

The C-terminal pentapeptide of C-peptide has shown to have the same activity as C-peptide in such assays as binding to cell membranes,[37] increasing Na^+/K^+-ATPase activity[39] and increasing intracellular Ca^{2+}.[40] The importance of the glutamic acid residue at position 27 (Glu27) in the amino acid sequence of C-peptide has also been proven to be vital in many biological assays.[39, 40] To validate the ability of the C-terminal pentapeptide to become activated by Zn-Ins and the importance of Glu27, the pentapeptide EGSLQ and its mutant peptide AGSLQ were used in the ATP assay with Zn-Ins. As shown in Figure 3.7, both wild type C-peptide and the pentapeptide EGSLQ significantly increased the ATP release of red blood cells. Even when Zn-Ins was used as the Zn^{2+} source, the activity of EGSLQ did not significantly change from that of the wild type. On the other hand, the mutant pentapeptide, AGSLQ, could not evoke a significant release of ATP from the cells.

PSS contains bovine serum albumin, which has a high affinity for Zn^{2+} ($K = 9.54 \times 10^7$). Therefore when Zn^{2+} activated C-peptide is added to PSS, serum albumin may remove the Zn^{2+} from C-peptide, decreasing the ability of C-peptide to stimulate ATP release from red blood cells. Studies in our lab have indicated that if C-peptide is surrounded with other ions like K^+, Na^+ or with bovine serum albumine (BSA) prior to mixing with Zn^{2+}, then its ability to increase the ATP release from RBCs is decreased by a considerable amount. To investigate how long the C-peptide Zn^{2+} complex can withstand an environment rich in BSA before it begins to lose its ability to stimulate cellular activity, PSS was added to Zn^{2+} and C-peptide mixed in water and the resulting solution was kept for varying times before adding the red blood cells.

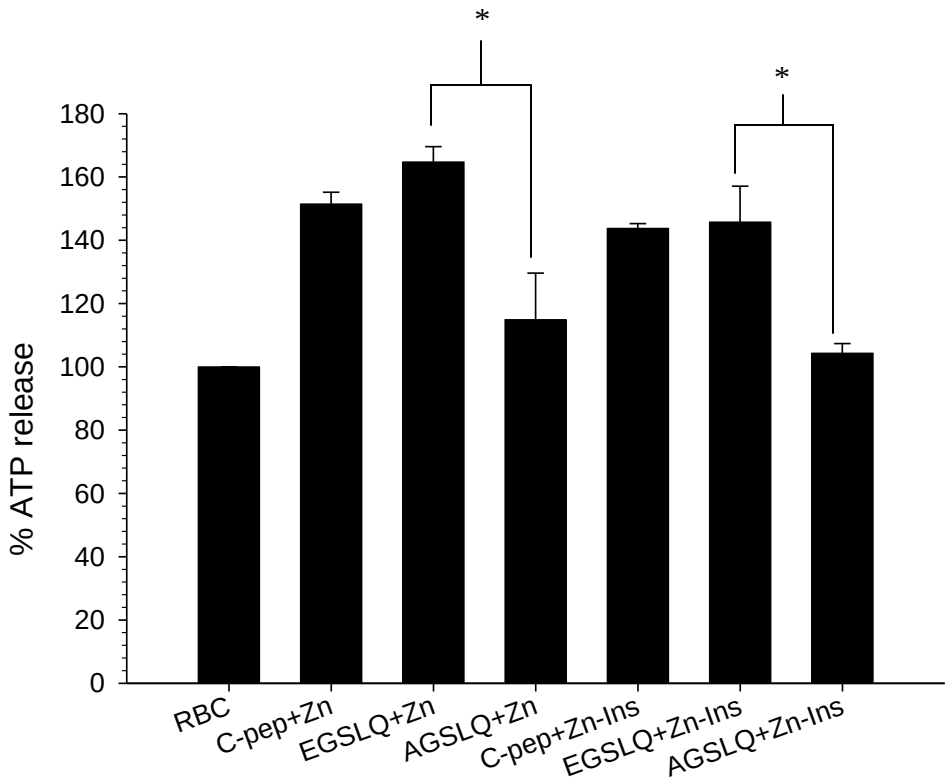

Figure 3.7: The normalized ATP release from red blood cells. The C-terminal pentapeptide EGSLQ showed the ability to increase the ATP release from red blood cells in the presence of $ZnCl_2$ and Zn-Ins. The mutant pentapeptide AGSLQ, with an alanine substitution, showed lowered ability in increasing the ATP release from red blood cells. Error bars ± SEM (n = 3), $p^* < 0.05$.

Figure 3.8 shows that the longer Zn^{2+} activated C-peptide is left to interact with PSS, the more C-peptide loses its ability to stimulate ATP release from red blood cells. During 5 min of exposure to PSS, ~20% decrease in C-peptide stimulated ATP release was observed, which further decreased by ~50% at the end of 1 hour exposure. This suggests that in order to evoke the maximum effect, Zn^{2+} activated C-peptide should reach the red blood cells before the Zn^{2+} is removed from the peptide.

Red blood cells from type 2 diabetic patients release less ATP when stimulated with Zn^{2+} activated C-peptide.[34] This effect seems to be due to red blood cells exposed to high glucose environment expressing more phophatidylserine (PS) on their outer membrane.[34] C-peptide being a negatively charged peptide at physiological pH might face repulsion from a more negatively charged cell membrane rendering the peptide unable to evoke any beneficiary effects.

To study the effect of high glucose on C-peptide-red blood cell interaction, red blood cells were pre-incubated in high glucose sugar (10 mM glucose and 20 mM glucose) for 2 hours at 37 ºC. The amount of C-peptide interacting with the pre-incubated red blood cells was calculated using an ELISA as mentioned earlier. From the results (Figure 3.9) it is evident that C-peptide interaction with the red blood cell decreases when cells were incubated at high glucose. At 20 mM glucose no significant interaction could be observed between the peptide and the red blood cell.

Similar results were observed during the ATP assay to observe the effect of C-peptide on ATP release from red blood cells pre-exposed to high glucose (15 mM glucose). When red blood cells, prior exposed to a high glucose environment, were incubated with Zn^{2+} activated C-

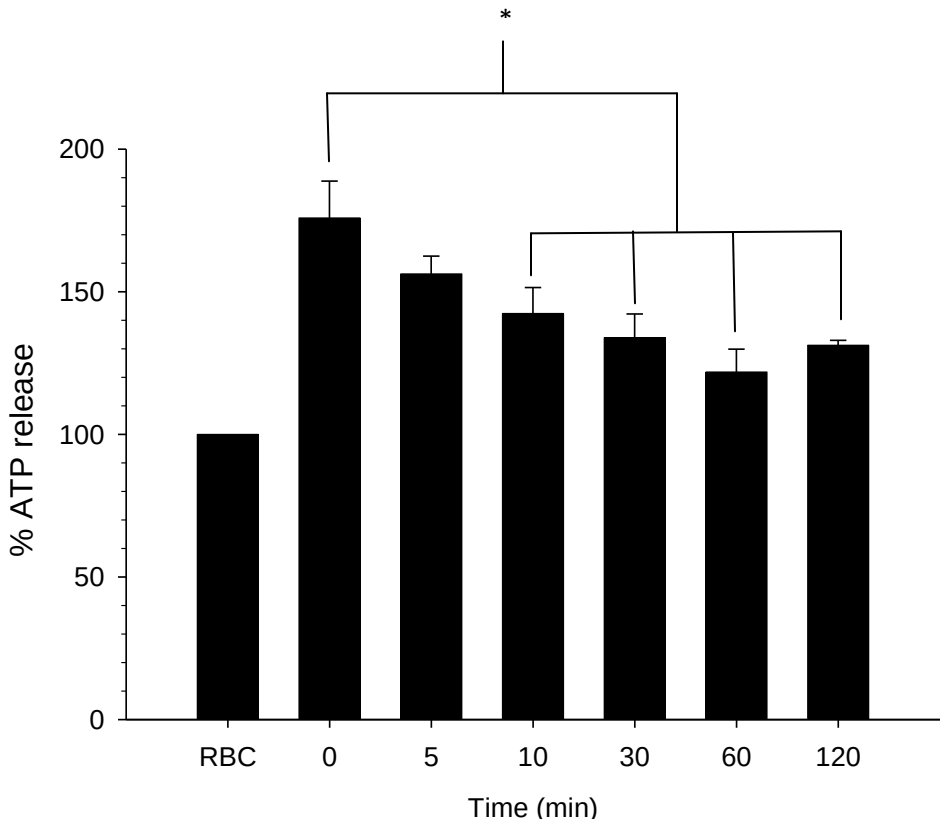

Figure 3.8: The normalized ATP release from red blood cells. Zn^{2+}-activated C-peptide was exposed to PSS for different time intervals before red blood cells were added. The maximum ATP release was obtained when there was no time lapse between addition of PSS and the red blood cells. ATP released decreased as a function of exposure time. Error bars ± SEM (n = 4), $p^{*} < 0.05$.

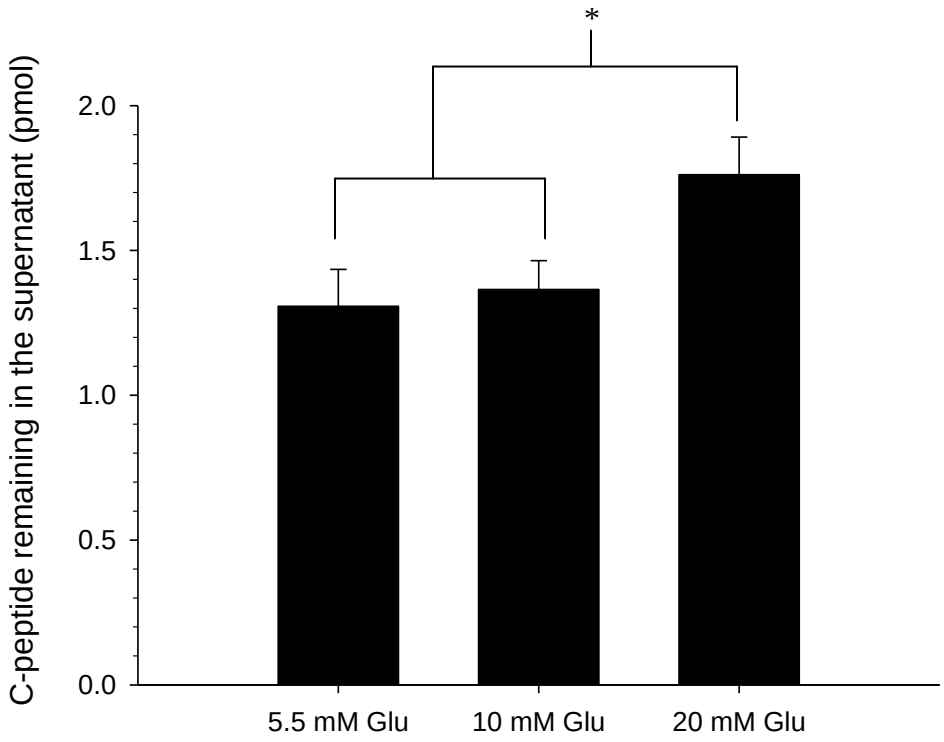

Figure 3.9: The C-peptide interaction with the red blood cells in normal and high glucose buffer. Total amount of C-peptide added to each sample is 2 pmol (2 nM). When red blood cells were incubated in high glucose PSS, the interaction between C-peptide and the cells decreased. Error bars ± std. dev. (n = 3), $p^{*} < 0.05$.

peptide, the ATP released increased only by 32 ± 5%, compared to the increase of 75 ± 10% in the cells in normal glucose environment (Figure 3.10). If the diabetic red blood cell membrane is interacting less with the negatively charged C-peptide due to the repulsion between a negatively charged cell membrane and the peptide, then binding of more Zn^{2+} to the peptide might overcome that repulsion. Results show that indeed more Zn^{2+} on the peptide facilitates increasing the C-peptide mediated ATP release of red blood cells incubated in a hyperglycemic environment to that of normal red blood cells. When the C-peptide:Zn^{2+} ratio was 1:1, hyperglycemic red blood cells released 42 ± 5% less ATP than normal cells. However, when the ratio was 1:3, there was no significant difference between the ATP release from the diabetic and normal RBCs.

However, it should be noted that with healthy red blood cells, the optimum effect of C-peptide was achieved at 1:1 ratio of Zn^{2+} to C-peptide. When the Zn^{2+} concentration was increased, the resulting ATP release started to diminish, and at 1:5 Zn^{2+} to C-peptide ratio, it decreased by ~20% relative to the 1:1 ratio. This may be due to the 1:1 ratio of Zn^{2+} to C-peptide is more effective in increasing Zn^{2+} uptake in red blood cells, and also in changing the C-peptide secondary structure. (Chapter 2, Figures 2.20 and 2.21)

3.4 Discussion

Although C-peptide had been considered a byproduct of insulin since its discovery, many beneficial effects of C-peptide in reducing diabetic complications have been reported during the past two decades.[30, 41, 42] To elicit a hormone-like effect, it is believed that C-peptide should

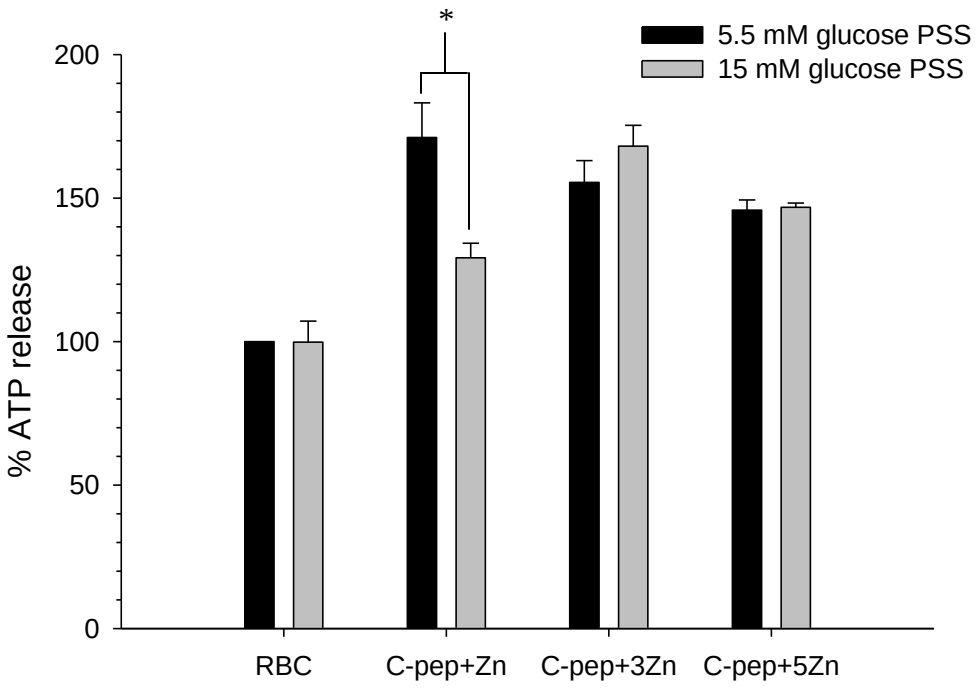

Figure 3.10: The normalized ATP release from red blood cells in normal and high glucose buffer. Red blood cells pre-exposed to high glucose PSS release lowered amount of ATP when incubated with Zn^{2+}-activated C-peptide. This could be overcome by adding more Zn^{2+} to the peptide. At normal glucose concentration, the optimum increase in ATP release was obtained when 1:1 ratio of Zn^{2+} to C-peptide was used to stimulate the red blood cells, Error bars are ± SEM (n = 4), $p^{*} < 0.05$.

bind to a receptor. However, efforts in isolating a cellular receptor for C-peptide have been unsuccessful.[36, 38] It has been suggested that the interaction between the peptide and the cell membrane is non-chiral, without any involvement of a receptor.[39, 43] Due to the ability of C-peptide to stimulate ATP release from red blood cells, some type of cellular interaction must be present between the peptide and the red blood cell. The ELISA assay that was performed in order to investigate the binding between C-peptide and red blood cell suggested that the amount of C-peptide binding to cells increased until the cells became saturated around 10 pmol of C-peptide (10 nM) added. At this point around 2 pmol of C-peptide was bound to the cells, or approximately ~1800 C-peptide molecules binding per cell. Another interesting factor is that when physiologically non-relevant higher concentrations (>10 nM) of C-peptide was added, the fraction bound started increasing revealing non-specific interactions under high concentrations.

A parallel study performed in the Spence lab using radioactive $^{65}Zn^{2+}$ shows very similar effects of C-peptide on $^{65}Zn^{2+}$ uptake in red blood cells (Figure 3.11). During the study, red blood cells were incubated with increasing amounts of $^{65}Zn^{2+}$-activated C-peptide. At the end of the incubation, the $^{65}Zn^{2+}$ uptake in the cells was measured using a liquid scintillation counter. Similar to the binding curve obtained for C-peptide, $^{65}Zn^{2+}$ uptake also increases with increasing additions of $^{65}Zn^{2+}$-activated C-peptide and becomes saturated around 10 pmol of Zn-C-peptide added to the cells (10 nM). Around 2 pmol of $^{65}Zn^{2+}$ were found on the red blood cells. Combined results from the two studies show that each C-peptide molecule interacting is capable of delivering a $^{65}Zn^{2+}$ ion to the cell.

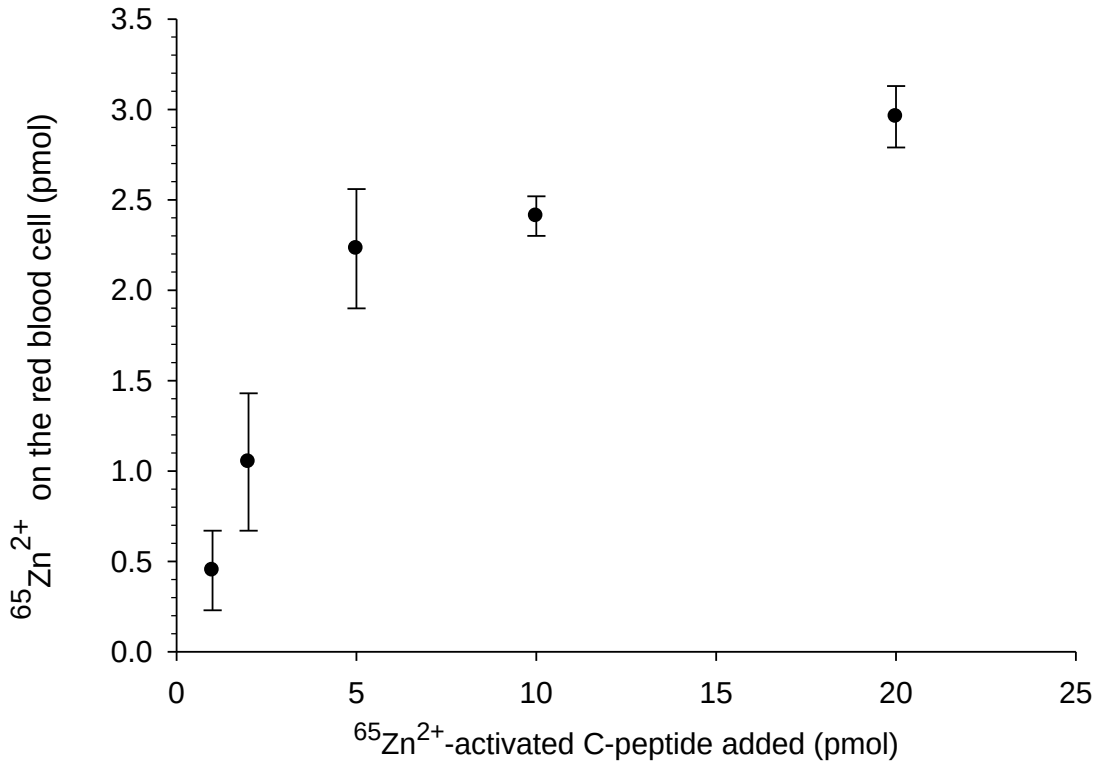

Figure 3.11: The $^{65}Zn^{2+}$ uptake in red blood cells as a function of $^{65}Zn^{2+}$-activated C-peptide added to 1 mL of 7% red blood cell sample. Curve shows a similar trend as that of C-peptide uptake as a function of Zn^{2+}-activated C-peptide added. When specific binding was considered, it was calculated that around 2 pmol of $^{65}Zn^{2+}$ was present on the red blood cells at saturation, giving a 1:1 ratio for C-peptide and $^{65}Zn^{2+}$ uptake. Error bars are ± std. dev. (n = 5).

When C-peptide was absent, there was no significant uptake of $^{65}Zn^{2+}$ in the red blood cells. Whether Zn^{2+} is interacting with the cell membrane or whether it is transported into the cell is still not clear.

Zn^{2+} influx in red blood cells has been reported, and the entrance of Zn^{2+} into red blood cells is suggested to occur through the Cl^-/HCO_3^- anion transporter.[44, 45] Being a cation, Zn^{2+} needs complexation with anions to cross the membrane. However, the Zn^{2+} concentrations used in these previous experiments were at µM-mM levels. (Interestingly, Zn^{2+} can increase ATP release from red blood cells at 1 mM concentration, without the any aid from C-peptide). However, such concentrations of Zn^{2+} are physiologically non-relevant.

From the $^{65}Zn^{2+}$ studies (data not shown), it is evident that C-peptide is required for the uptake of Zn^{2+} in to red blood cells at low nM concentrations of Zn^{2+}. To determine whether Zn^{2+} is essential for C-peptide interaction with cells, red blood cells were incubated with C-peptide and Zn^{2+} activated C-peptide. The difference in interaction was studied with an ELISA assay. Surprisingly, our data suggests that C-peptide binding to red blood cells does not depend on the presence of Zn^{2+}. This shows that C-peptide can interact with the red blood cell even in the absence of Zn^{2+}. However, Zn^{2+} is necessary to evoke an increase in cellular metabolism and ATP release.

Although the elucidation of a cellular receptor for C-peptide has not been successful, several studies have shown the ability of C-peptide to bind to a variety of cells. Rigler *et.al.* used fluorescence correlation spectroscopy to demonstrate the ability of C-peptide to bind to renal tubular cells, skin fibroblasts and saphenous vein endothelial cells.[36] Among these, renal tubular cells showed the highest density of binding sites for C-peptide, 75 sites per μm^2. However, endothelial cells from umbilical cord veins did not show any C-peptide binding. This is interesting considering that C-peptide increased nitric oxide (NO) synthase in aortic endothelial cells, but had no effect on umbilical endothelial cells.[36] Renal tubular cells demonstrated a high binding affinity for C-peptide ($K = 3.3 \times 10^9\ M^{-1}$), and there was no competition for binding from insulin, insulin like growth factor-I, proinsulin or the scrambled or nonnative D-C-peptide. This indicates specific ligand-receptor interaction of C-peptide with renal tubular cells. Interestingly, C-terminal pentapeptide showed the ability to displace bound C-peptide from the cells, indicating that the interaction may be through the C-terminal region of the C-peptide. The binding was abolished when the cells were pre-treated with pertussis toxin, which modifies receptor coupled G-proteins, indicating that the mechanism of C-peptide action may be through interaction with a G-protein coupled receptor. In addition, C-peptide has also shown interactions with solubilized cell membranes.[46] During the interactions with renal tubular cells, Glu27 of the C-terminal pentapeptide proved the most essential, while the last three amino acids (Ser29, Leu30, Gln31) proved to be intermediately essential.[37] The rest of the peptide (1-26 portion) had no effect on binding.

The interaction of Zn^{2+}-activated C-peptide increases the ATP release from red blood cells and endothelial cells.[35] The presence of the metal ion proved necessary for this activity. From the results shown in figure 3.6, it is evident that the source of Zn^{2+} is inconsequential, and the activity of C-peptide on red blood cells is not affected by the presence of insulin. However, a high affinity metal chelator such as EDTA completely abolishes the activity of the peptide. Figure 3.7 shows that the C-terminal pentapeptide EGSLQ has similar ability to increase ATP release from red blood cells as that of wild type C-peptide when activated by Zn^{2+} or Zn-Ins. The mutant peptide AGSLQ had significantly lower activity, confirming the necessity of Glu27 for the stimulation of the ATP release. The lack of ATP release by AGSLQ, combined with binding affinities of the mutants in chapter 1, suggests that Glu27 is necessary for binding with Zn^{2+}, as well as for the biological effect of the peptide on the red blood cell. The importance of Glu27 for the stimulation of red blood cell derived ATP release by Zn^{2+}-activated C-peptide was also reported by Keltner *et. al.* using Zn (II) chloride as the Zn^{2+} source.[47]

ATP is the universal source of chemical energy for living cells.[48] An extracellular role of ATP was not accepted at first, due to the belief that the cells would not release a molecule so fundamental for life. The size and charge of the molecule, which restricts simple diffusion out of the cell, supported this idea.[48] However, it was later discovered that ATP is released from a variety of cells, especially neuronal cells,[49] and cells of the vascular system such as platelets,[50] endothelial cells,[51] smooth muscle cells[51], mononuclear cells[52] and red blood cells.[13] In non-

neuronal cells a large number of stimuli are responsible for ATP release, including (but not limited to) agonists, hypoxia, acidosis, mechanical deformation and receptor stimulation.[13, 15, 50, 53] Red blood cells, apart from being the oxygen carriers in the circulation, can serve as regulators of local blood flow.[12] Earlier it was believed that this regulatory effect of red blood cells is through delivery of NO, bound to hemoglobin, to peripheral tissues.[54] But kinetic studies have shown that this method of vasorelaxation is only possible in the smallest of microvessels.[55] Thus, attention shifted to the release of ATP form red blood cells having regulatory effect on the blood flow. Red blood cells from humans[13] as well as other species[12, 15, 17] have shown the ability to release ATP when exposed to hypoxia,[12, 13] acidic pH,[12] mechanical deformation[15, 17] and other chemical stimulations such as iloprost[56] and metal-activated C-peptide.[33] More importantly, the release of ATP from red blood cells has been directly related to the regulation of vascular flow.[57] The increase in perfusion seems to be mediated via an endothelial-derived NO dependent vasorelaxation.[57-59] The vasorelaxation due to ATP can be abolished by applying L-NAME, an endothelial NO synthase (eNOS) inhibitor.[57, 60]

Stimuli such as acetylcholine,[61] bradykinin,[62] and ATP[63] result in an increase in NO synthesis in endothelial cells. Endothelial cells primarily express P_{2y} receptors for binding ATP,[64] and this binding of ATP to the receptor stimulates eNOS by increasing intracellular Ca^{2+}.[65, 66] The binding of Ca^{2+}-calmodulin complex to eNOS increases the NO production.[67]

NO released from endothelial cells causes vasodilation via relaxation of adjacent smooth muscle cells (figure 3.12). NO results in heme-dependent activation of cytosolic guanylate cyclase (GC) and cyclic GMP (cGMP) accumulation in vascular smooth muscle cells. cGMP causes relaxation in vascular smooth muscle, most probably by intracellular binding of free calcium, resulting in lowering of free calcium concentration, leading to muscle relaxation.[68] NO also has an inhibitory effect on platelet aggregation[69] and platelet endothelial interaction,[70] again reducing the risk of vascular complications.

The regulatory effect of the red blood cell on blood flow is often impaired in diabetic patients. Exposure to a high glucose environment can have adverse effects on red blood cell properties.[71-73] Red blood cells from uncontrolled diabetic patients have impaired deformability compared to healthy humans.[74] Red blood cells obtained from healthy humans, when exposed to high glucose environment, also showed impaired deformability.[75] In response to a high glucose environment, red blood cells can form sorbitol via the aldose reductase pathway.[76, 77] Bareford *et.al.* showed a direct relationship between intracellular sorbitol concentrations and red blood cell deformability.[71] It was suggested that the increased sorbitol, which cannot diffuse out of the cell, affects the osmotic properties and deformability of the cells. Decreased deformability of red blood cells can have an impact on the red blood cells traversing through microvasculature, and it also affects the amount of ATP released from the cells. This can lead to impaired blood rheology.

An alternate explanation for decreased deformability of erythrocytes is the glycosylation of red blood cell membrane proteins,[78, 79] which again can increase membrane rigidity.[80] Alterations in red blood cell membrane lipid composition have also been observed in diabetic

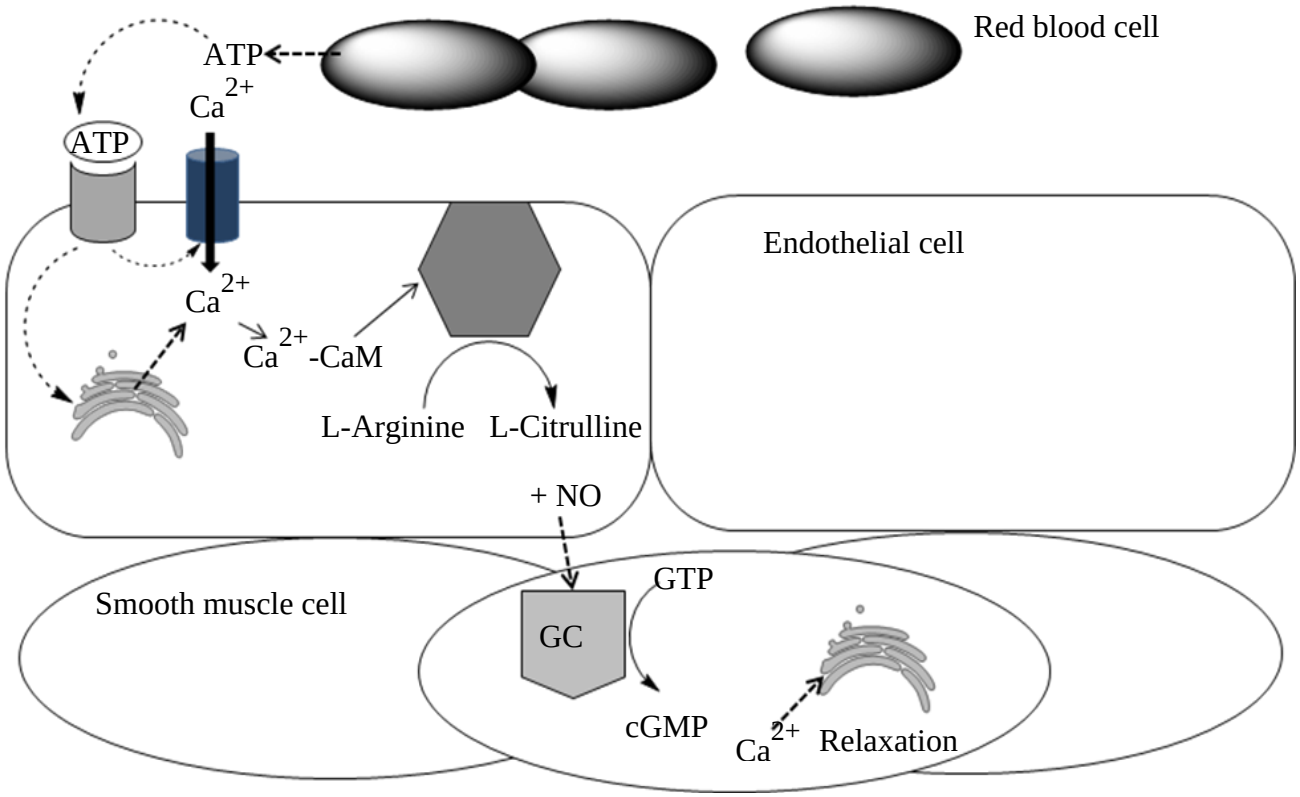

Figure 3.12: Proposed mechanism for the ATP stimulation of endothelial NO synthesis and the resultant smooth muscle cell relaxation. ATP binding to P_{2y} receptors on endothelial cells results in an increase in intracellular Ca^{2+}. Ca^{2+}, via Ca^{2+}-calmodulin (Ca^{2+}-CaM) stimulates endothelial NO synthase (eNOS). NO diffuses to the smooth muscle cell and causes vasodilation through a cyclic GMP (cGMP) mediated mechanism.

patients. The increased cholesterol to phospholipid ratio was linked to decreased membrane fluidity in these calls.[81, 82]

Red blood cells of diabetic patients also have an impaired defense mechanism against oxidative stress.[83] Glutathione (GSH), responsible for keeping the cellular proteins from oxidation, is maintained at a high concentration in the red blood cell. GSH protects the sulfhydryl group of cystein in proteins from oxidation,[84, 85] and also protects the cell against oxidation from free radicals and reactive oxygen intermediates.[86] A decrease in the concentration of GSH has been reported in red blood cells from diabetic patients.[87] A decrease in glutathione synthesis, in addition to decreased activity of glutathione reductase, seems to be the reason for the lowered GSH level.[83] The oxidation of membrane cytoskeleton proteins such as spectrin can lead to decreased red cell deformability.[88, 89] The weakened defense system against oxidative stress can lead to decreased levels of deformation-induced release of adenosine triphosphate (ATP) from erythrocytes in diabetic patients.[8]

The phospholipids of the cell membrane are asymmetrically distributed between inner leaflet and outer leaflet of healthy cell membranes, phosphatidylcholines (PC) predominantly in the outer leaflet and phosphatidylserine (PS) in the inner leaflet.[90] In patients with diabetes mellitus, this equilibrium can be disrupted exposing more PS molecules on the outer leaflet of the membrane.[91] These patients also had elevated levels of scramblase enzyme, which is responsible for the externalization of PS. The exposure of PS can increase cardiovascular risk of diabetes

patients by increasing red blood cell adhesion. PS also controls membrane charge, and can determine protein interactions with the membrane.[92]

Previous studies in Spence group have shown the decrease in the ability of metal-activated C-peptide to induce ATP release from red blood cells from type 2 diabetic patients, in comparison to red blood cells from healthy controls.[34] As C-peptide is negatively charged at physiological pH, it was suggested the exposure of PS in red blood cell membrane hinders the interaction between the peptide and the red blood cell. This effect could be overcome by addition of metformin, a therapeutic agent used to treat type 2 diabetic patients. The role of metformin was suggested to be the masking of charge repulsion between cell membrane and C-peptide.

The study discussed in figure 3.9 shows that red blood cells incubated in high glucose PSS, showed less interaction with metal-activated C-peptide. This also affected the metal-activated C-peptide induced ATP release from red blood cells (Figure 3.10). If the loss of interaction between peptide and red blood cell is due to repulsion, then it may be overcome by adding more Zn^{2+} to the medium. Figure 3.10 shows that when three times excess Zn^{2+} was added, the release of ATP from cells incubated in high glucose PSS was not significantly different from that of red blood cells incubated with regular glucose PSS. However, $^{65}Zn^{2+}$ studies show that 1:1 Zn^{2+} to C-peptide ratio is optimum for Zn^{2+} uptake in red blood cells (Figure 2.15). The decrease in ATP release at higher Zn^{2+} to C-peptide ratios can be due to this effect.

Although the beneficial effects of C-peptide have been constantly reported, several drawbacks of C-peptide against its use as a therapeutic agent in diabetes mellitus have also been

discussed.[38] One such argument is that in type 2 diabetic patients, although C-peptide is present during the early stage of the disease, the complications of the disease still develop as in type 1 patients. However, this statement is not completely true, as it has been reported in many complications such as retinopathy and neuropathy that progression in type 1 patients is more rapid than in type 2 patients.[93, 94] In addition, from the results discussed in this chapter it can be suggested that cells exposed to high glucose environment, such as in uncontrolled diabetes, can show 'resistance' to C-peptide. This shows that even though C-peptide is present in the circulation, it might not be able to show optimal effect.

Another drawback of C-peptide that has been discussed is the lack of long-term successful clinical trials.[38] Again, this might be due to the discrepancies in C-peptide administration. Several publications from the Spence group have discussed the necessity of a metal ion such as Zn^{2+} for optimum and reproducible bio-activity of C-peptide.[33-35] In addition, to obtain optimum activity, the metal-activated C-peptide should not be exposed to serum albumin or high concentrations of other competing metal ions. Figure 3.8 shows the effect PSS has on the activity of Zn^{2+}-activated C-peptide. The results suggest that, to obtain reproducible results, freshly prepared metal-activated peptide should be added to the cells. Shafqat *et.al.* observed that when insulin and C-peptide were administered through the same port to the patient, the decrease in glucose level was significantly higher than in separate administration of insulin and C-peptide via different ports.[95] The authors credited this observation to the ability of C-peptide to break down the Zn-insulin hexamer, however, another possibility is the 'activation' of C-peptide in the process and the resulting increase in metabolism caused by Zn^{2+}-activated C-

peptide. Therefore, it can be suggested that C-peptide administration in the presence of Zn^{2+} may lead to reproducible clinical trials.

Many reports have indicated the importance of C-peptide in ameliorating vascular complications of diabetes mellitus. These beneficial effects mostly seem to have arisen by stimulating endothelial-derived NO synthesis.[30] In addition, C-peptide treatment also increased Na^{+}/K^{+}-ATPase activity in red blood cells.[30, 31] Both these effects can be linked to increased production and release of ATP in the cells. The increase of ATP production in turn can be linked to increased metabolism, which can explain the increase in glucose utilization observed after C-peptide administration.[96, 97] Higher glucose utilization was observed in red blood cells *in vitro* by Meyer *et. al.* when the cells were incubated in metal-activated C-peptide.[33] Many cell types that have shown C-peptide sensitivity, such as red blood cells, endothelial cells, renal cells and neuronal cells, have insulin independent glucose transporters. Although red blood cells are reported to have insulin receptors,[98] it is evident that insulin does not have any effect on the red blood cell derived ATP release (Figure 3.6). Therefore, it is possible that most of the effects of C-peptide are evoked through increased metabolism via increased insulin-independent glucose uptake. The mechanism behind this effect and the role of Zn^{2+} cannot be completely explained yet. It is interesting that Zn^{2+} has shown the ability to control cell membrane fluidity[99] and non-insulin dependent glucose uptake.[100] According to data presented in this chapter, it can be suggested that Zn^{2+} plays a role in increasing glucose metabolism in red blood cells.

It has been reported that the glucose transport in red blood cells in type 2 patients is impaired, and the activation energy needed for glucose uptake was increased.[101] The reason for this was concluded to be a structural change in the glucose transporter GLUT1, although it remains to be discovered whether Zn^{2+}-activated C-peptide has a direct effect on the glucose transport via GLUT1.

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Chapter 4 – Overall Conclusions and Future Directions

4.1 Overall Conclusions

For nearly 30 years after its discovery C-peptide was mainly regarded as a byproduct of insulin synthesis. Later, evidence began to emerge indicating the ability of C-peptide to reduce diabetic complications such as neuropathy,[2, 3] nephropathy,[4, 5] retinopathy[6] and microvascular complications.[7-9] People with type 2 diabetes, who have circulating insulin and C-peptide, develop diabetic complications slower than type 1 patients,[10, 11] suggesting a possible importance of C-peptide in avoiding diabetic complications. Although people with diabetes mellitus receive exogenous insulin, they eventually develop complications of the disease. Therefore, controlling hyperglycemia alone is not enough to avoid all diabetic complications.[12, 13]

The ability of C-peptide to reduce vascular complications is important because it leads to a decrease in other complications such as retinopathy, neuropathy and nephropathy. C-peptide administration increases flow-mediated vasodilation[14] and improves blood flow.[15, 16] The observed beneficial effects are believed to be mediated via the ability of C-peptide to increase endothelial derived NO release,[8, 9] deformability of red blood cells,[17] and red blood cell Na^+/K^+-ATPase activity.[8, 18] The molecule that may be playing a major role in controlling these effects of C-peptide is ATP (adenosine triphosphate). ATP is a stimulus for the production of endothelial-derived NO,[19] which is a major determinant of vasorelaxation, leading to improved

blood flow.[20] Therefore, it can be suggested that the initial effect of C-peptide is to increase cellular metabolism and ATP production and/or release. In agreement with this hypothesis, C-peptide administration significantly increased glucose metabolism in patients with type 1 diabetes.[21-23]

In continuance, Meyer *et. al.* were the first to report the ability of C-peptide to increase ATP release from rabbit red blood cells.[24] However, for this action to occur, C-peptide required the presence of a metal ion such as Fe^{2+} or Cr^{3+}. Due to the fact that Zn^{2+} is the most abundant metal ion in the pancreas, later studies were conducted in the presence of Zn^{2+}.[25, 26] The need for metal addition to C-peptide is supported by the fact that some of the reported C-peptide effects were only observed in the presence of insulin (insulin hexamers contain Zn^{2+}).[13, 27] Optimum glucose utilization in type 1 patients was observed when C-peptide and insulin were administered together at the same port.[23] In addition, some effects were reported to be abolished in the presence of EDTA, a strong metal ion chelator.[28, 29]

In order for the metal ion to evoke C-peptide activity, an interaction between C-peptide and the metal ion is required. An initial characterization of this interaction, the binding between C-peptide and Zn^{2+}, was studied with the aid of a Zn^{2+} fluorescent probe TSQ (N-(6-Methoxy-8-quinolyl)-p-toluenesulfonamide). Using Scatchard plots, the binding constant and maximum number of Zn^{2+} ions binding to the peptide were calculated. It was observed that the binding between Zn^{2+} and C-peptide changes with pH. At pH 5.5, Zn^{2+} binds to C-peptide with a higher

binding constant ($K = 1.22 \times 10^7\ M^{-1}$), than at pH 7.4 ($K = 7.99 \times 10^6\ M^{-1}$). In mature pancreatic β-cell granules, the pH is approximately 5.5; therefore, C-peptide has the ability to bind to Zn^{2+} inside the granule and subsequently release this Zn^{2+} upon interaction with the red blood cell at physiological pH (pH 7.4). Interestingly, we have observed that for optimum effect, C-peptide and Zn^{2+} had to be mixed in deionized water (pH ~5.5), prior to addition of physiological salt solution (PSS) and red blood cells.

The number of Zn^{2+} ions binding to the C-peptide also varies with pH. At pH 5.5 ~1 Zn^{2+} ion is bound whereas at pH 7.4 ~2 ions are bound. The fact that only one ion binds at lower pH, with a comparably higher binding constant, led us to believe that (a) there is more than one residue involved in the binding and (b) there may be chelation between the carboxylate residues and the Zn^{2+}. Circular dichroism (CD) studies were carried out to investigate any structural changes incorporated with this chelation. It was observed that at a 1:1 Zn^{2+} to C-peptide ratio, the overall randomness of the chain decreased, as indicated by the change in the minimum in ellipticity around 200 nm in the far-UV CD spectrum. This was not observed at a 5:1 Zn^{2+} to C-peptide ratio. However, there were no changes in the spectrum that would suggest formation of any secondary structure such as an α-helix or β-sheet.

Some portions of the C-peptide sequence have been proven to be essential for the bio-activity of the peptide. C-peptide has five acidic amino acid residues, including four glutamate residues and one aspartate residue. The glutamate residue at position 27 (Glu27) has been reported to be essential for many cellular effects of C-peptide.[30, 31] In addition, the glutamate

residues at positions 3 and 11 are also important.[30, 32] Keltner *et. al.* reported the necessity of Glu27 for Zn^{2+} binding and also for the stimulation of ATP release from red blood cells.[33] During our study, five single amino acid mutants of C-peptide, where one of the acidic amino acids was substituted with an alanine, were studied to compare their binding affinity with Zn^{2+}. When comparing the binding constants, the wild type C-peptide had the highest binding constant and the Glu27, Glu3 and Glu11 mutants (E27A, E3A and E11A, respectively) resulted in the lowest binding constant. This shows that the substitutions at the positions 3, 11 and 27 have the greatest effect on Zn^{2+} binding. When considering the number of Zn^{2+} ions bound, the wild type peptide showed the lowest number (~1) while E27A had the highest (~4). The higher number of Zn^{2+} ions on E27A with a lower binding constant may suggest an open structure for this mutant. The mutation at position 1 (E1A) seemed to have the least effect on Zn^{2+} binding.

Although the efforts by previous groups to identify a cellular receptor for C-peptide have been unsuccessful, several studies have shown the ability of C-peptide to bind to cells.[34, 35] To stimulate cellular glucose uptake and ATP release, it would seem likely that Zn^{2+}-activated C-peptide should have some kind of cellular interaction with the red blood cell. An enzyme linked immunosorbant assay (ELISA) was used to determine the amount of C-peptide interacting with red blood cells. When 1 mL of a 7% red blood cell sample was incubated with increasing amounts of Zn^{2+}-activated C-peptide, it was observed that the maximum amount of C-peptide interacting with the red blood cells was at around 2 pmol of Zn^{2+}-activated C-peptide (1:1 ratio), or ~1500 molecules per red blood cell. In renal tubular cells, this number is reported to be around

~1000-1500 molecules per cell.[34] Incidentally, the number of insulin receptors reported to be present on the red blood cell is ~2000.[36] These interaction numbers reveal that C-peptide has some kind of interaction with the red blood cell, which may enable it to evoke the stimulatory effect on the red blood cell derived ATP release. However, the nature of this interaction and whether a specific receptor is involved is yet to be determined, although with the new knowledge presented in this dissertation involving Zn^{2+}, this quest for a receptor should be revisited.

It was hypothesized that Zn^{2+} binds with the C-peptide and enables it to interact with the red blood cell. To examine this, C-peptide, in the presence and absence of Zn^{2+}, was added to red blood cells and the interaction was measured using the ELISA. Surprisingly, the amount of C-peptide binding to the cells was independent of Zn^{2+}. This indicates that the interaction between the healthy red blood cell and C-peptide did not require the presence of Zn^{2+}. A parallel study performed in the Spence group (Suzanne Letourneau) using $^{65}Zn^{2+}$-activated C-peptide showed that $^{65}Zn^{2+}$ uptake by red blood cells was only observed in the presence of C-peptide. The $^{65}Zn^{2+}$ uptake showed a similar trend to the C-peptide uptake, reaching a saturation point around 2 pmol of $^{65}Zn^{2+}$ for a 1 mL, 7% red blood cell sample. This data strongly suggests a 1:1 ratio of C-peptide to Zn^{2+} was interacting with the cells. Therefore, it seems that C-peptide serves as a carrier for Zn^{2+} ions and increases the amount of Zn^{2+} in the red blood cells. No significant effect on ATP release is observed with C-peptide in the absence of metal ion, suggesting that Zn^{2+} is involved in increasing glucose uptake and ATP release from red blood

cells. However, the exact mechanism behind this is not yet fully understood. Interestingly, when the E27A mutant was used instead of the wild type C-peptide, the $^{65}Zn^{2+}$ uptake decreased, showing that the glutamate residue at position 27 is necessary for the C-peptide mediated Zn^{2+} uptake in red blood cells.

Further studies with $^{65}Zn^{2+}$-activated C-peptide showed that a 1:1 ratio of Zn^{2+} to C-peptide was required for delivery of the $^{65}Zn^{2+}$ uptake into the red blood cells when the PSS buffer contained bovine serum albumin (BSA). BSA, which has a high affinity for Zn^{2+} ($K = 9.54 \times 10^{7}$), is capable of removing loosely bound Zn^{2+} from C-peptide. However, at the 1:1 Zn^{2+} to C-peptide ratio, the Zn^{2+} ion is held with higher affinity and the 1:1 complex is able to deliver the Zn^{2+} ion to the red blood cell, even in the presence of BSA. In the absence of BSA, complexes with higher ratio of Zn^{2+} to C-peptide were able to deliver Zn^{2+} to the red blood cell, although typically saturating at ~ 2 pmol. This also agrees with the CD studies where only the 1:1 complex induced a change in the spectrum, indicating there is a reduction in randomness of the peptide chain.

Until now, only the Spence group has used a metal ion in C-peptide assays and claimed it necessary to have the metal ion for C-peptide activity on red blood cells. However, other research groups have reported the necessity of insulin for the success of C-peptide studies.[13, 27] Insulin hexamers contain Zn^{2+}, which may aid in C-peptide activity. To observe this effect, insulin containing Zn^{2+} (Zn-Ins) and insulin without Zn^{2+} (Ins) were added to C-peptide instead

of $ZnCl_2$, and the resulting ATP release from red blood cells was measured. As expected, in the presence of Zn-Ins, C-peptide stimulated ATP release increased by 44 ± 11%, while in the presence of Ins it increased by only 15 ± 9%. The addition of $ZnCl_2$ as the Zn^{2+} source led to an increase in ATP release of 64 ± 12%. Zn-Ins alone had no effect on ATP release from red blood cells. This indicates that Zn^{2+} added to C-peptide in the form of Zn-Ins can lead to activation of C-peptide, which may explain the successful C-peptide trials reported in the presence of insulin.

The C-terminal pentapeptide has shown similar ability as the full length C-peptide in many assays and cell binding studies.[34, 37] Importantly, this is the region containing the Glu27 residue. To investigate the importance of the pentapeptide and Glu27, the C-terminal pentapeptide EGSLQ and its single amino acid mutant AGSLQ were investigated instead of the full length C-peptide and the resultant ATP release was determined. EGSLQ was able to increase the ATP release from red blood cells both in the presence of $ZnCl_2$ and Zn-Ins. It was not significantly different from the effect of the full length peptide. The ATP release from AGSLQ treated red blood cells, both in the presence of $ZnCl_2$ and Zn-Ins, was not significantly different from that of untreated red blood cells. This indicates that the C-terminal pentapeptide has the ability to elicit similar effects to the full length C-peptide in the presence of Zn-Ins, but for proper activity, the presence of the glutamate residue at position 27 is necessary.

There are several arguments against C-peptide being a potential therapeutic agent against diabetic complications. Namely, a) the receptor for C-peptide has not been identified, b) patients with type 2 diabetes, having circulating C-peptide, still develop complications of the disease

similar to type 1 patients who do not have C-peptide in their circulation, and c) no successful long-term clinical trials have been reported.[38]

Meyer et.al. have shown that red blood cells obtained from type 2 diabetic patients released significantly lower amounts of ATP when incubated with Zn^{2+}-activated C-peptide in comparison to red blood cells obtained from healthy, non-diabetic donors. This led to the hypothesis that red blood cells exposed to high sugar develop 'resistance' to C-peptide, which may explain why C-peptide could not prevent these patients from developing diabetic complications. This resistance was suggested to be associated with the exposure of negatively charged phosphatidylserine (PS) on the outer leaflet of the red blood cell membrane.[25] Metformin, a drug used to treat type 2 diabetes, helps to overcome the resistance and increase the Zn^{2+}-activated C-peptide stimulated ATP release from diabetic red blood cells to that of control red blood cells. It is assumed that metformin, positively charged at physiological pH, will help reduce the repulsion between negatively charged peptide and the cell membrane, although this has yet to be confirmed.[25]

To further explore the effect of C-peptide in hyperglycemic environments, rabbit red blood cells were exposed to high glucose prior to incubation with Zn^{2+}-activated C-peptide. It was observed that red blood cells incubated in normal glucose PSS (5.5 mM glucose) showed an increase in ATP release of 71 ± 12% after stimulation with Zn^{2+}-activated C-peptide, while cells incubated in high glucose PSS only showed an increase of 29 ± 5%. This indicates that exposure to high glucose creates a 'resistance' to C-peptide stimulation. If this decrease in C-peptide effect

is due to PS exposure, it was hypothesized that the addition of excess Zn^{2+} can help in overcoming the repulsion, similar to the effect by metformin. After addition of 1:3 C-peptide to Zn^{2+} ratio, the ATP release from red blood cells incubated in high and normal glucose sugar PSS were not significantly different from each other, indicating excess Zn^{2+} was beneficial in ameliorating the 'resistance'. However, the ATP release at higher Zn^{2+} to C-peptide ratios (3:1 and 5:1) in normal glucose PSS were lower than that observed at the 1:1 Zn^{2+} to C-peptide ratio. This may be due to the fact that the 1:1 Zn^{2+} to C-peptide complex is more efficient in stimulating Zn^{2+} uptake into the healthy cells, as observed by the $^{65}Zn^{2+}$ studies.

Taken collectively, the results indicate that the effect of C-peptide on the red blood cell depends heavily not only on the presence of the metal ion (Zn^{2+}), but also on the correct ratio of Zn^{2+} to C-peptide and the pH at which Zn^{2+} and C-peptide are mixed. The effect also seems to rely on the amount of glucose to which the red blood cells are exposed. As discussed in chapter 3, to achieve the optimum effect on ATP release, C-peptide and Zn^{2+} had to be mixed in deionized water and the red blood cells had to be added immediately after the addition of PSS. A lapse between the addition of PSS and red blood cells resulted in a decrease in ATP release from stimulated red blood cells.

The data presented in this dissertation lead to the overall conclusion that, in order to achieve best and reproducible results from C-peptide administration, care should be taken to create optimum conditions for binding between Zn^{2+} and C-peptide. Also immediate interaction

between Zn^{2+}-activated C-peptide and red blood cells is desirable to obtain the optimum effect. These findings may help in achieving successful and reproducible clinical trials for C-peptide.

4.2 Future Directions

Data presented here suggest that the use of Zn^{2+}-activated C-peptide to treat diabetic complications may have beneficial effects on the patients. However, more studies are needed to validate the potential of Zn^{2+}-activated C-peptide as a therapeutic agent. For example, how Zn^{2+}-activated C-peptide is involved in the mechanism of stimulation of ATP release from red blood cells is not yet fully understood. Two hypotheses that can be suggested about this mechanism are: (a) C-peptide serves as the carrier for Zn^{2+}, and Zn^{2+} uptake results in the increase in ATP production and release and (b) the binding of Zn^{2+} enables the C-peptide to bind to the cell correctly, and leads to increased ATP release. Due to the fact that C-peptide interaction with the cell is unaffected by the presence of Zn^{2+}, the former hypothesis seems to be the most likely. However, the possibility that the Zn^{2+}-C-peptide complex evokes an effect cannot be excluded.

The deformation induced pathway for ATP release from the red blood cell has been studied and a mechanism has been suggested by Sprague (Figure 4.1).[1] A G-protein coupled receptor (GPCR) is involved in the mechanism. Interactions of C-peptide with cells such as renal tubular cells,[34] and opossum kidney proximal tubular cells[39] have been linked to a GPCR mediated mechanism.

Therefore it seems possible that the Zn^{2+}-activated C-peptide is also acting via this receptor. However, unpublished studies performed by previously in the Spence lab show that inhibition of GPCR or adenylyl cyclase (AC) did not have a profound effect on the metal-activated C-peptide

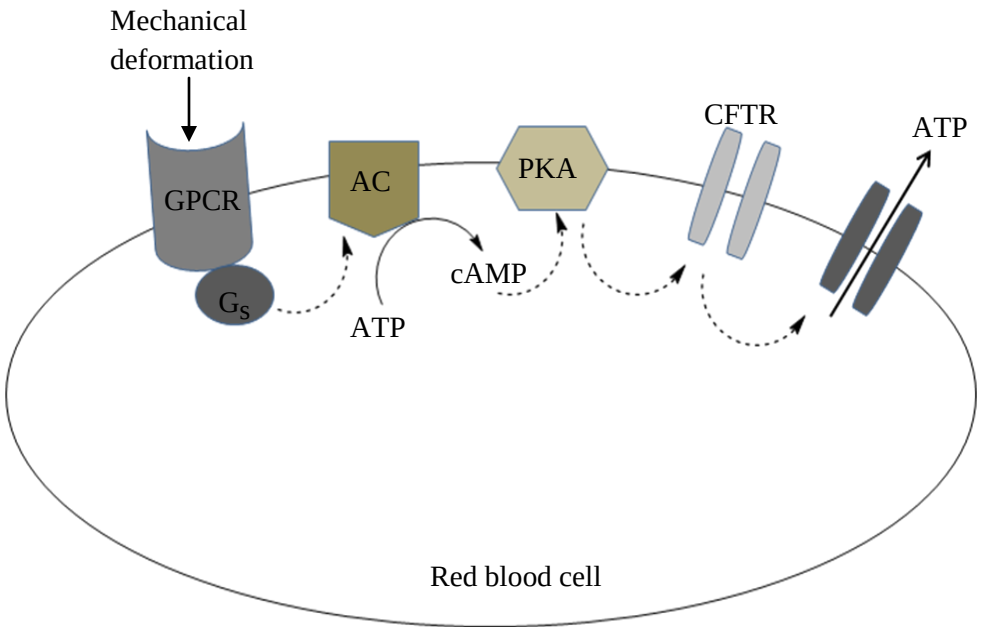

Figure 4.1: The proposed mechanism for the deformation-induced ATP release from the red blood cells.[1] GPCR - G-protein coupled receptor, AC – adenylyl cyclase, PKA – protein kinase A, CFTR – cystic fibrosis transmembrane conductance regulator. Although some cellular effects of C-peptide are suggested to follow a G-protein coupled receptor pathway, the stimulatory effect of C-peptide on ATP release from red blood cell seems to occur through a different signal mechanism.

induced ATP release of red blood cells. However, the inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) did have an effect on the ATP release, indicating that the ATP release is CFTR mediated, in agreement with previous studies by Sprague.

Metal-activated C-peptide can increase the glucose uptake in the red blood cells.[24] Inhibition of CFTR decreased the glucose uptake, indicating the cell regulates the metabolism according to the amount of ATP that can be released. Because C-peptide does not act through a GPCR, and it affects the glucose uptake in the red blood cells, this led to the suggestion that Zn^{2+}-activated C-peptide may mediate its effect through an increase in the activity of the red blood cell glucose transporter, GLUT1.GLUT1 is a facilitative glucose transporter found in many types of cells. GLUT1 is abundant in red blood cells,[40] endothelial cells (especially in the blood-brain barrier and the eye),[41, 42] and epithelial cells in the eye.[42] It is also expressed in lower levels in virtually all tissues.[43] GLUT1 is a non-insulin dependent glucose transporter. The function of GLUT1 has been known to be tightly regulated by response to hormonal and metabolic signals.[44] In pigeon red blood cells, hypoxia increased the glucose uptake several folds without increasing the amount of GLUT1 in the plasma membrane.[45] In muscle cells and adipocytes, insulin increases GLUT4 in the cell membrane.[46] However, the increase in glucose uptake is higher than that accounted for by the increase in membrane GLUT4, indicating there is also an increase in the intrinsic activity of GLUT4 and GLUT1. The exact mechanism behind the stimulation of glucose transport is currently unknown.[47]

Group IIB metal ions (Cd^{2+}, Zn^{2+}, Hg^{2+}) have shown the ability to stimulate glucose uptake in rat adipocytes.[48] Among these metal ions, Cd^{2+} has been further studied and shown to increase the intrinsic activity of glucose transporters in 3T3 L1 adipocytes.[49] In an *in vitro* study conducted by Lachaal *et. al*., Cd^{2+} increased the glucose binding affinity of GLUT1, suggesting a metal ion induced conformational change in the transporter.[47] The Cd^{2+} interaction was believed to occur via cysteine residues in GLUT1, which do not participate in glucose binding.[47]

Zn^{2+} has shown the ability to increase non-insulin-dependent glucose uptake in both healthy human subjects[50] and cirrhosis patients.[51] Again, the effect was suggested to be via enhanced glucose transporter activity. The effect of Zn^{2+} or the Zn^{2+}-C-peptide complex in evoking an increase in glucose uptake and ATP release from red blood cells may be caused by increased activity of GLUT1 in these cells. To determine whether Zn^{2+}-activated C-peptide has a direct effect on the glucose transporter in the red blood cell, the interaction of Zn^{2+} and Zn^{2+}-C-peptide with GLUT1 and the change in the glucose affinity of the transporter should be studied.

In order to observe the interaction between GLUT1 and Zn^{2+} or Zn^{2+}-C-peptide in vitro, GLUT1 was purified from red blood cell membrane ghosts as previously described.[52] The procedure was followed with slight modifications. The proteins were separated in the presence of decylmaltoside at pH 6.0, using an anion exchange column. The chromatogram is shown in Figure 4.2.

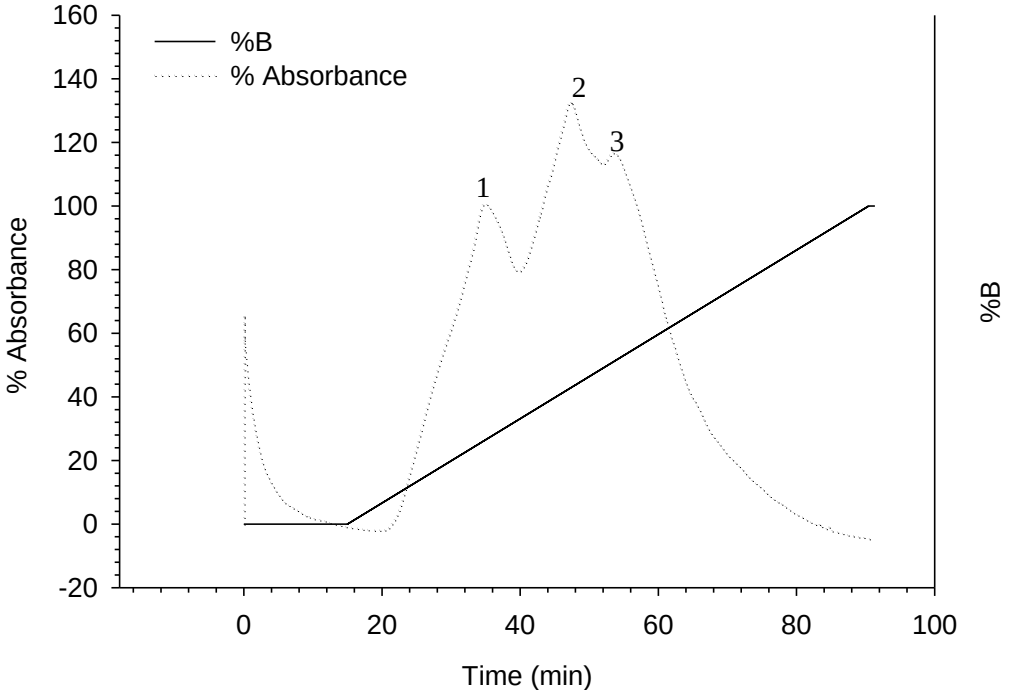

Figure 4.2: Chromatogram showing the separation of protein fractions from anion exchange chromatography. Solvent A is 10 mM Bis-tris containing 0.05 mM EDTA and 0.2% decylmaltoside. Solvent B is solvent A with 0.5 M NaCl. A 5 mL QTrap column was used for separation. The protein fractions were eluted at a flow rate of 0.25 mL/min. 0.5 mL fractions were collected. The fractions were concentrated and identified by gel electrophoresis. Fraction 1 contained GLUT1.

The protein fractions were collected and gel electrophoresis (SDS-PAGE) was used to analyse the fractions for GLUT1 (Figure 4.3). GLUT1 elutes around 100 mM NaCl concentration in ion exchange chromatography and appears as a broad band between 49 to 64 kD in gel electrophoresis (The molecular weight of 55 kDa changes with the degree of glycosylation). Fraction 1, which contained the band between 49-64 kDa, was identified as containing GLUT1 and subsequent studies were performed using fraction 1.

The protein fraction containing GLUT1 was washed three times using BSA-free PSS to remove the excess EDTA and decylmaltoside in the eluting buffer used during separation. The protein concentration of the fraction was determined using a bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL). An aliquot of the protein was incubated with $^{65}Zn^{2+}$, and the amount of $^{65}Zn^{2+}$ binding to the protein was determined using molecular weight cut-off filters (3 kDa) and liquid scintillation counting. After washing with BSA free PSS, only about 8% of the protein fraction was bound to $^{65}Zn^{2+}$, at 50 nM $^{65}Zn^{2+}$ concentration. It is expected that at this low concentration, Zn^{2+} will not interact with GLUT1 without the aid of C-peptide. The next step would be to incubate $^{65}Zn^{2+}$-activated C-peptide with the GLUT1 fraction and observe if there is an increase in $^{65}Zn^{2+}$ binding to GLUT1.

The GLUT1 fraction can be further characterized by using a western blot with anti-GLUT1 antibodies, which will confirm the presence of GLUT1. GLUT1 can also be characterized by binding with cytochalasin B, a competitive inhibitor of GLUT1.[53] A MS analysis can also be performed for identification.

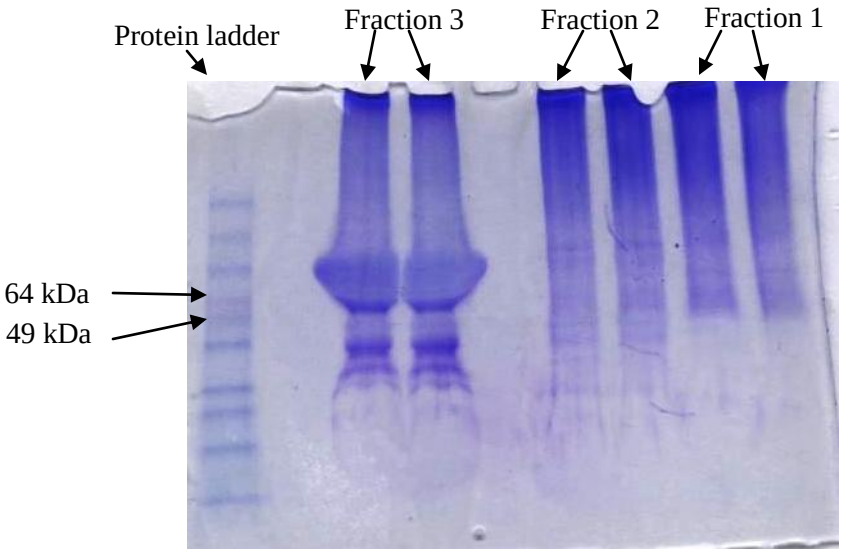

Figure 4.3: Gel electrophoresis (SDS-PAGE) to analyze the fractions collected from the anion exchange chromatography. Fraction 1 contained the typical broad protein band of GLUT1 between 49-64 kDa. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

The binding of radio-labeled glucose to GLUT1 in the presence and absence of Zn^{2+}-activated C-peptide can be measured. This will show whether or not Zn^{2+}-activated C-peptide has an effect on the substrate affinity of GLUT1.

C-peptide interaction with the red blood cell was studied using an ELISA as discussed in chapter 3. C-peptide has shown the ability to be internalized into the cells.[54] Therefore, the amount of C-peptide interacting with the cell as calculated in chapter 3 may be an overestimation if some of the C-peptide enters the cell allowing more C-peptide to bind to the cell membrane. The absolute binding of C-peptide to the cell membrane can be investigated by using sealed red blood cell ghost membranes, instead of intact cells, during the study.

Protective effects of Zn^{2+} on the cell membrane have been described. Zn^{2+} is important in maintaining the membrane integrity,[55] and deficiency of Zn^{2+} can increase osmotic fragility of the cell.[56] Therefore, Zn^{2+} can have an indirect effect on red blood cell membrane ATP release through control of the cell membrane properties. The antioxidant properties of Zn^{2+} help protect the proteins and lipids in cell membranes and cytoskeleton form oxidation, maintaining the cell deformability.[57] As mentioned in previous chapters, cell membrane deformability is a major determinant of ATP release from the cell.[58] Therefore the effect of Zn^{2+} uptake on membrane properties should be studied in order to determine if it has a direct effect on cellular ATP release. One such parameter that can be studied is the amount of cholesterol in the cell membrane. The amount of cholesterol is a determinant of cell membrane rigidity, and consequently, cell deformability. There is an inverse relationship between Zn^{2+} and cholesterol in the cell

membrane.[59] The amount of cholesterol in the cell membrane as a function of added Zn^{2+}-activated C-peptide can be studied in order to determine if the acute effect of Zn^{2+}-activated C-peptide on ATP release is due to loss of cholesterol from cell membrane.

After being exposed to a high glucose environment, red blood cells show 'resistance' to the effect from Zn^{2+}-activated C-peptide (chapter 3). Incubation of red blood cells with metformin helps overcome this effect.[25] It has been suggested that metformin optimizes the cellular interaction between Zn^{2+}-activated C-peptide and red blood cells exposed to high glucose.[25] This can be studied using the ELISA. Preliminary data in our lab support the hypothesis that in the presence of metformin, diabetic red blood cells interact more with Zn^{2+}-activated C-peptide.

To date, we have only reported studies on a limited number of cell types. The effect of C-peptide on red blood cells, endothelial cells and macrophages (unpublished data) all need the presence of a metal ion such as Zn^{2+} for optimum results.[26] However, the variety of effects shown by C-peptide on various types of cells suggest the need to investigate the overall effect of C-peptide in more detail. Some studies have reported the need of the presence of insulin for proper activity of C-peptide.[13, 27] Work by Shafqat *et.al.* suggest that administration of C-peptide and insulin at the same port is requisite for optimum glucose clearance, again indicating the need of insulin together with C-peptide for activity.[23] These experiments suggest that either C-peptide enhances insulin action or insulin is providing the metal-activation for C-peptide due

to its contamination with Zn^{2+}. All these observations indicate more thought should be given to proper administration and presence of a metal ion may be needed in order to achieve reproducible results in C-peptide treatment. It would be beneficial to repeat some of the cited studies with purified C-peptide (to ensure no metal ion contamination) in the presence and absence of a metal ion such as Zn^{2+}. This would provide definitive evidence on whether or not metal-activation of C-peptide is a major requirement for all cellular effects of C-peptide. It also may help explain the failure of some C-peptide studies and lack of successful clinical trials.

It would also be of interest to use radio-labeled C-peptide to study the interaction between C-peptide and intact cells/cell membranes. Although the same amount of C-peptide interacts with red blood cells in the presence or absence of Zn^{2+} (chapter 3), the binding affinities of the cell to C-peptide may be different in the two cases. Displacement studies using radio-labeled C-peptide may provide further insight.

In vivo studies are needed to further evaluate the effect of Zn^{2+}-activated C-peptide on healthy and diabetic animal models. This will help understand the effect of Zn^{2+}-activated C-peptide on glucose utilization and blood flow in real time, and also to determine the optimum parameters in terms of C-peptide administration with Zn^{2+}.

Finally, other metal ions with reported potential to an increase in glucose uptake, such as Cr^{3+} and Cd^{2+}, should be studied to study the ability to bind to C-peptide and to evoke a change in cellular metabolism in comparison to Zn^{2+}. Although Zn^{2+} has the best chance to bind to C-

peptid*e in vivo*, the possible combination of a different metal ion with C-peptide may help to obtain prolonged and improved activity of C-peptide both *in vitro* and *in vivo*.

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