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Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships

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INTRODUCTION

Insulin is a 51 amino acid anabolic peptide-hormone that is secreted by the β -cells in the Islets of Langerhans. Insulin consists of two chains (A and B) connected by disulfide bonds. One of its primary functions is the stimulation of glucose uptake from the systemic circulation, as well as the suppression of hepatic gluconeogenesis, thereby serving a primary role in glucose homeostasis and preventing the metabolic disorder diabetes mellitus (1,2,3,4,5,6). Diabetes is characterized by decreased glucose tolerance resulting from a relative deficiency of insulin or a lack of sensitivity to the endogenous hormone. Insufficient insulin, or decreased insulin sensitivity, results in hyperglycemia which is associated with the development of complications such as vascular disease, particularly coronary heart disease, cerebrovascular disease, and the characteristic retinopathy, nephropathy, and neuropathy of this disorder (7,8).

It was Von Mering and Minkowski that first noted that removal of the pancreas led to the development of diabetes mellitus in dogs (9). However it was Schafer in 1916 that first speculated that the antidiabetic hormone, which he decided to call "insuline," was from the pancreatic islets (4). Barron noted in 1920 that ligation of the pancreatic duct, with destruction of the exocrine pancreas, only resulted in diabetes if the islets, named by Dr. Langerhans in 1869 (10), were also destroyed (11). Subsequently, the work of Banting, Best, Collip and MacCleod in the early 1920's resulted in the identification of a substance in extracts of pancreas that had the remarkable ability to reduce blood glucose levels in diabetic animals (12,13); and by 1923 these pancreas extracts were being used to successfully treat diabetic patients. Insulin regulates a host of other cellular processes, such as protein and fat synthesis, RNA and DNA synthesis, as well as cell growth and differentiation. It is, however, the regulation of glucose uptake that is of primary concern in the clinical manifestations of diabetes; therefore, this chapter will begin with a brief description of how plasma glucose homeostasis is achieved.

REGULATION OF PLASMA GLUCOSE BY INSULIN

It is now known that specific membrane transporters facilitate the movement of glucose into cells to reduce plasma glucose concentrations in response to insulin stimulation. The transported glucose is subsequently used as metabolic fuel or stored as the complex macromolecular structure, glycogen. Two major types of glucose transporters are known: Na⁺-dependent and Na⁺-independent transporters. Only the Na⁺-independent transporters possess an

insulin responsive isoform. The Na⁺-dependent glucose transporter has been identified in several tissues particularly in the small intestine epithelium and the proximal tubule cells of the kidney, as well as in other kidney tubule cells (14,15,16). These transporters are located on the luminal side of intestinal and kidney cells and act to absorb glucose against its concentration gradient by coupling the movement of glucose into these cells with the concomitant movement of Na⁺ into the cell. Since Na⁺ is moving down its electrochemical gradient this energy can be used to cotransport glucose into the cells. Thus, this transporter is dependent on the concentrations of extracellular and intracellular sodium which are maintained by a Na⁺/K⁺-ATPase ion pump.

The Na⁺-independent glucose transporter family consists of several isoforms which facilitate the movement of glucose down its concentration gradient across a plasma membrane. Although seven isoforms have been identified (Glut1-7) (17) only one will be discussed in detail here, Glut4, because it is the transporter that is in highest concentration in insulin-sensitive tissues such as, skeletal muscle, fat, and cardiac muscle (18,19,20). Glut4, and to a lesser extent Glut1 enable these cells to increase their glucose uptake, thereby lowering circulating glucose levels. Because the intracellular concentration of glucose is low due to the rapid phosphorylation of glucose to glucose-6-phosphate and its dissimulation to other metabolic products, the presence of active transporters in the plasma membrane favors the movement of glucose into cells.

Insulin enhances glucose uptake by increasing the number of transporters in the plasma membrane of cells. This was first demonstrated in adipocytes (21,22) and subsequently in skeletal (23,24) and cardiac muscle (25). Insulin stimulation of cells mobilize transporters from intracellular compartments to the plasma membrane to facilitate glucose transport. This translocation of receptors to the plasma membrane has been demonstrated to occur within 30 seconds of insulin stimulation (26) and as the stimulus dissipates the decrease in the number of plasma membrane receptors declines coincident with a decline in glucose transport (27). While glucose transport via Glut4 is a passive process limited only by the chemical potential of the glucose gradient and the V_{max} of the transporters, translocation and reverse reinternalization of receptors are energy dependent processes (22).

The impaired ability of insulin to signal Glut4 translocation from intracellular stores is currently believed to be an important contributory factor to postprandial hyperglycemia in diabetes (28). Animal studies have also demonstrated that insulin resistance is associated with a decreased translocation of glucose transporters to the plasma membrane in muscle cells (30). In fact, decreased insulin levels in diabetic animals have been shown to not only decrease transporter translocation but diminish expression of Glut4 in muscle cells (31,32). Thus, it appears that insulin serves not only to acutely increase glucose transporter translocation, but also to maintain a basal level of expression of transporters in cells. Thus one mechanism by which diabetes, characterized by either low insulin levels, as in type 1 diabetes, or insulin resistance, as in type 2 diabetes, could cause pathologically high plasma glucose levels is via loss of regulation and expression of transmembrane glucose transporters. Several authors have also proposed that Glut2 on the β-cell membrane is relevant in regulating insulin secretion from islets (33,34). Accordingly, a β-cell specific insulin receptor knock-out (KO) model indicated that insulin likely positively regulates its own secretion from the β-cell (35).

The central importance of insulin in regulating glucose metabolism and the prevention of diabetes has stimulated research over 80 years in attempts to understand the mechanism of action of this peptide hormone. This work has led to the determination of the three dimensional structure of insulin, identification of its precursor and the processing and secretion mechanisms that underlie its production, as well as, identification of the insulin receptor and of its mechanisms of signal transduction. Moreover, these discoveries have added to our understanding of the molecular basis of diabetes and its treatment, as will be discussed in greater detail in this section, as well as in other sections in this chapter.

INSULIN BIOGENESIS AND MECHANISM OF RELEASE

Insulin was the first peptide hormone discovered. Before Abel crystallized insulin in 1926 (36), and Jensen and Evans hydrolyzed the N-terminal phenylalanine of the B-chain (37), proving that insulin was indeed a protein, all hormones were believed to be small molecules. With the elucidation of the primary sequence of insulin by Sanger in the mid 1950's (6,38) (see Figure 1a) it became known that insulin was a two chain heterodimer consisting of a 21 amino acid A-chain linked to a 30 residue B chain by two disulfide bonds derived from cysteine residues (A7-B7; and A20-B19). An intrachain disulfide bond also exists in the A-chain (A6-A11). Although this primary structure provided valuable information on the amino acid composition and size (?6000 D) of the insulin molecule (39), questions concerning the processes of insulin synthesis and secretion were not resolved until the late 1960's with the discovery of proinsulin – the precursor of insulin (40,41). Proinsulin, a 9 kdalton protein, contains both the A- and B-chain of insulin in a continuous single chain joined through an intervening region called the C-peptide (41,42). The C-peptide is a variable length peptide segment, consisting of 26-31 residues depending on the species, which links the carboxy terminus of the B-chain to the amino terminus of the A-chain via two dibasic residue links (Arg-Arg and Lys-Arg) as shown in Figure 1b. Proinsulin is cleaved at those dibasic links by a trypsin-like enzyme to release two chain insulin and free C-peptide.

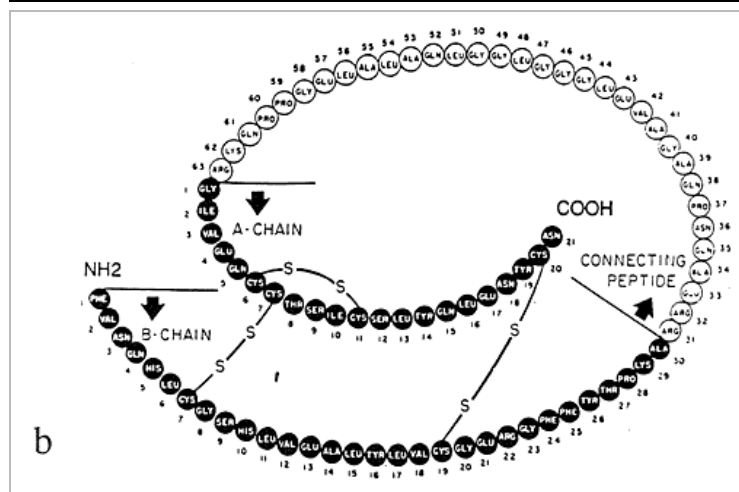
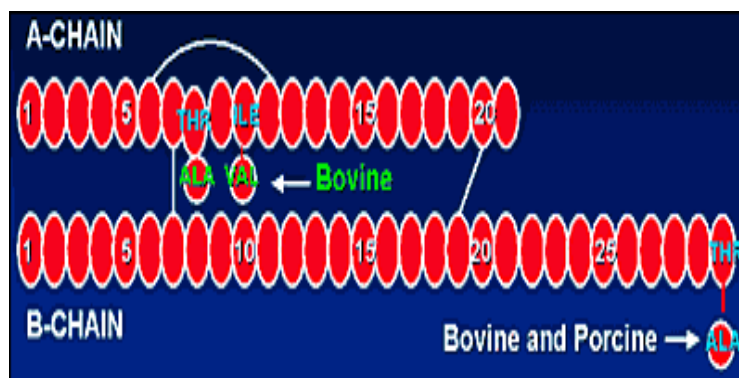
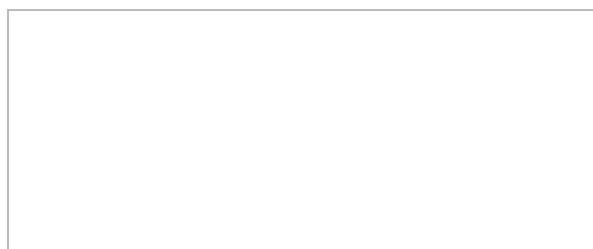


Figure 1. Primary structures of porcine insulin and porcine proinsulin. The primary sequence of porcine insulin (a) as determined by Sanger and co-workers (Ref 231); and proinsulin (b). The sequence of human insulin is identical to that of porcine insulin except for the change of AlaB30 to ThrB30 in human insulin.

Chan et al. (43) subsequently discovered that there was an additional precursor of insulin, proinsulin. Proinsulin is a 12 kdalton single chain polypeptide which consists of proinsulin extended at the amino terminus by a 24 amino acid signal peptide region of hydrophobic residues (44,45). This signal sequence is characteristic of proteins that enter the secretory pathway (46,47,48). Figure 2 illustrates the steps involved in the conversion of the information encoded within the 1500 bases of the human insulin gene sequenced by Bell et.al. in 1980 (49), into proinsulin and its proteolytic conversion to insulin. The initial mRNA transcript is modified via excision of the two intervening sequences, capping of the 5' terminus by 7-methyl guanosine, and polyadenylation of the 3' terminus to produce a mature mRNA product. This mRNA product codes for proinsulin, which is translated on the rough endoplasmic reticulum (RER) and subsequently translocated into the RER lumen via a series of interactions of the signal peptide with the signal recognition particle (SRP) and the SRP-receptor in the RER membrane (50,51,52). The signal peptide is then cleaved off in the lumen of the RER by a signal peptidase located on the luminal side of the RER membrane (51). Within the cisternae of the RER proinsulin undergoes rapid folding and disulfide bond formation to generate the native tertiary structure of proinsulin, the direct precursor of insulin (Figure 3). Proinsulin is then transported to the Golgi apparatus where it is packaged into secretory granules and then converted to native insulin and C-peptide. The signal peptide is rapidly degraded in the RER and is therefore not a normal secretory product of the β -cells (53). The conversion process may begin in the trans Golgi network but continues in the condensing vacuoles (early secretory granules), and the products are stored in mature secretory vesicles, and secreted in equimolar amounts along with small amounts (3%) of proinsulin and intermediate cleavage products (54). Glucose, in addition to stimulating insulin secretion, is also a direct stimulator of insulin gene transcription, and insulin mRNA translation and stability (55) in the β cells.



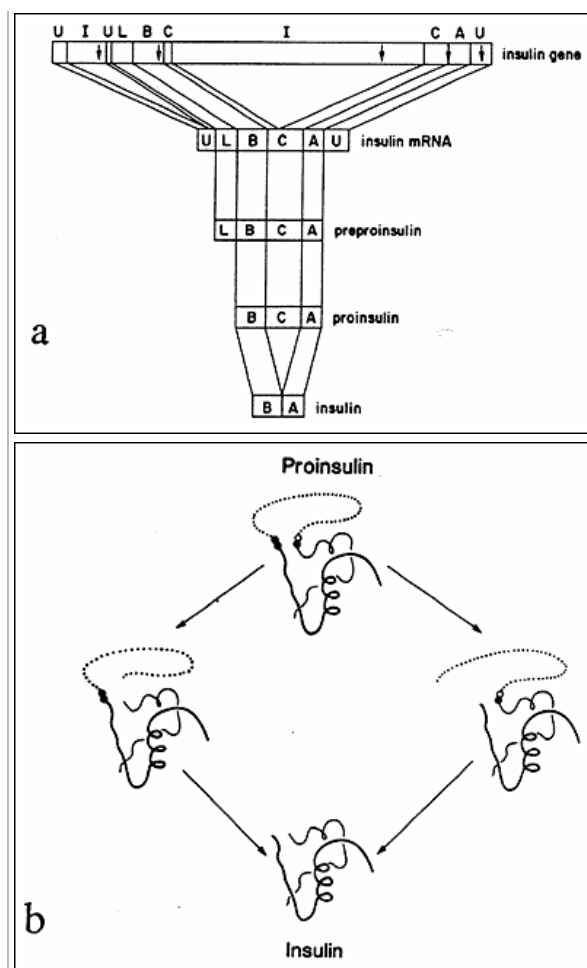


Figure 2. Diagrammatic illustration of the processing of insulin. The transcription and translation of the human insulin gene, as well as processing of preproinsulin to insulin is illustrated in (a). The conversion of proinsulin to insulin is illustrated in (b) demonstrating the secondary structure of insulin.

Proinsulin, the direct precursor of insulin, has many of the physical properties of insulin despite the larger size of this molecule. Proinsulin has been shown to aggregate, forming dimers and Zn^{2+} -coordinated hexamers in a manner similar to insulin (56), have a comparable isoelectric point (57,58,59) and solubility (54), as well as, react with insulin antisera (42,60). This prompted the suspicion that the structure of insulin in proinsulin is similar, if not identical, to that of native insulin. It was also found that proinsulin is a full agonist of insulin and displays 3-5% biological activity (61,62); therefore the binding regions of insulin are accessible even when the relatively large C-peptide is linked to insulin. The full 3-D structure of proinsulin has not been determined despite its successful crystallization (63,64) possibly due to the lack of a fixed orientation of the C-peptide, however its insulin moiety has appeared to be very similar to that of crystalline insulin (65).

In 1969, it was demonstrated via pulse chase studies that proteolytic processing of proinsulin occurred in the Golgi apparatus and/or early secretory vesicles of the β -cells; and subsequent studies have identified the trans Golgi as the initial site where proinsulin and its converting enzymes are brought together to form secretion granules (41,50,66,67). Monoclonal antibodies specific for intact proinsulin also demonstrated that proinsulin is transferred from the RER to the cis and to the trans Golgi where it is concentrated into prosecretory vesicles (68). Several studies have demonstrated that the shuttling of proinsulin vesicles from the RER to the cis Golgi and through the Golgi apparatus is an energy requiring process which requires ATP (66,67,69). The subsequent conversion of proinsulin to insulin is initiated in the trans Golgi and proceeds for several hours within secretory granules as they mature in the cytosol in preparation for secretion. Figure 3 shows the intracellular pathway taken by proinsulin upon budding from the RER, and gives the times required for individual stages of transfer. In rat islets the conversion of proinsulin to insulin begins about 30 min after synthesis and resembles first order reaction kinetics having a half-time of approximately 30-60 minutes (40,70).



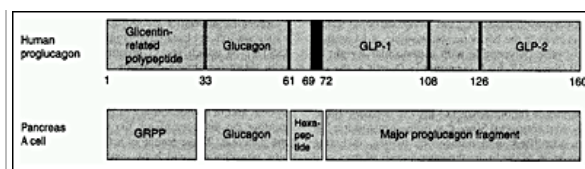


Figure 3. Insulin biosynthesis and secretion. Model of the intracellular synthesis, processing, and transport of preproinsulin, proinsulin, and insulin. Preproinsulin is synthesized in the RER and is converted to proinsulin within 1-2 min. Proinsulin is folded in the RER and transported to the Golgi apparatus. Clathrin-coated granules bud from the trans-Golgi contain proinsulin and the converting enzymes to cleave the prohormone to insulin. For further explanations see text.

The conversion of proinsulin to insulin occurs by the joint action of two types of proteases: one with trypsin-like endoprotease activity to cleave at the dibasic residues at each end of the C-peptide, and another with exopeptidase activity like that of carboxypeptidase B to remove the basic residues left after tryptic cleavage. Previous studies have also demonstrated that mixtures of pancreatic trypsin and carboxypeptidase B could convert proinsulin to insulin *in vitro* (71). Two endoproteases were found in insulinoma secretory granules and were initially called Type I and Type II (72). Both of these endoproteases were found to be Ca²⁺ dependent. Type I was active in 1 mM Ca²⁺ and cleaved at Arg31-Arg32 in proinsulin, and Type II required 0.1 mM Ca²⁺ and cleaved predominantly at Lys64-Arg65 (73). Both of these enzymes were also found to have a pH optima near 6.0, with Type II also functioning in the neutral range.

The discovery in yeast of an endoprotease known as Kex2 that cleaved at dibasic residue sites in the α -mating factor and killer toxin precursors facilitated the search for homologous proteases in mammalian β -cells (74,75,76). Kex2, a homologue of subtilisin, a bacterial serine protease, is an integral membrane protein and is localized in the trans Golgi network in yeast cells. Analysis of human insulinoma cDNA via PCR techniques lead to the discovery of PC2, an enzyme having a similar catalytic domain, (77). PC2 shared 49% amino acid identity with Kex2, but importantly lacked the transmembrane (TM) segment of Kex2, indicating that it was likely a soluble protein and a candidate for one of the processing endoproteases in β -cell secretory vesicles. Similar screening methods lead to the discovery of PC3/PC1, the second secretory granule convertase (78,79). Subsequent work also demonstrated that both PC2 and PC3/PC1 display optimal activity at pH 5.5 which is the internal pH of the β -cell secretory granules (80,81).

Further studies using immunocytochemical techniques on β -cells, and coinfection with vaccinia viruses expressing PC2 and PC3/PC1 in Cos7 cells expressing proinsulin, identified PC2 and PC3/PC1 in pancreatic islets and demonstrated their ability to convert proinsulin to insulin by selectively cleaving at the C-peptide junctions (82). PC2 demonstrated cleavage only at the C-peptide/A-chain junction, however, PC3/PC1 cleaved at both dibasic sites with a preference for the B-chain/C-peptide site. Later studies also established the identity of PC2 and PC3/PC1 with the calcium dependent Type II and Type I insulinoma proteases, respectively, discovered by Davidson et al (83). The carboxypeptidase B-like exopeptidase which removes COOH-terminal basic residues after cleavage by PC2 and PC3/PC1 was also found and is known as carboxypeptidase H (84). Carboxypeptidase H has several unique features which differentiates it from the other carboxypeptidases (85); however it has been shown to be structurally homologous to pancreatic carboxypeptidases A and B (86). Thus, working together in the maturing secretory granule of the B cell PC2, PC3/PC1, and carboxypeptidase H serve to convert proinsulin to mature insulin, and C-peptide.

As mentioned earlier, secretory granules in the β -cell undergo maturation in the cytosol. Electron microscopic studies have demonstrated that mature granules have a dense crystalline-appearing core with a spacing similar to that of 2-Zn insulin crystals (87,88,89). Studies suggest that newly synthesized insulin probably forms crystals with zinc that is transported into secretory granules; and these reside in the dense core of the β -granules with the soluble C-peptide in the less dense or clear periphery of the granule (89). Proinsulin is also known to crystallize with insulin in small amounts, probably as mixed hexamers (90). It is possible that the passage of zinc into the β -granules may be passive since both proinsulin and insulin have the ability to bind zinc (91,92,93) and form zinc coordinated hexamers having two Zn²⁺ atoms per hexameric unit. The side chain of histidine B10 of each insulin molecule is known to coordinate with zinc, stabilizing the hexamer. The secretory granules also actively exchange and take up specific ions (94), and possess an intrinsic proton pump which serves to lower the pH of the granule to pH 5.0-5.5 which is optimal for both conversion and crystallization *in vitro* (67,95,96). By contrast thiol-disulfide exchange is favored by the more alkaline pH of the RER which promotes proinsulin folding and disulfide bond formation.

Glucose, which is a positive effector of biosynthesis, is the primary regulator of insulin biosynthesis and secretion but other hormones and chemical substances also play an important role (97). Glucose causes an increase in cAMP levels by a mechanism which does not appear to involve activation of adenylate cyclase (98). cAMP then exerts its effects via a mechanism involving protein kinase A, leading to the phosphorylation and activation of certain key proteins (99). Through this complex chain of events, glucose and cAMP rapidly increase translation and transcription of insulin mRNA (100,101). Insulin mRNA normally turns over slowly, with a half life of approximately 30 hours at normal or below normal levels. However, elevated glucose increases the half life of insulin mRNA as much as three fold

(102,103). Calcium dependent exocytosis of secretory granules is the main mechanism of secretion in both glucose-stimulated and basal states (40,104,105). It should also be noted that little, if any, direct secretion of proinsulin occurs from the RER to the plasma membrane by way of any other unregulated pathway (105,106). Other stimulators of insulin secretion include glucagon, which is secreted by α -cells in the pancreas and is discussed in detail in another chapter; glucagon-like peptide (GLP-1: Ref. 107); cholecystokinin (108); and gastric inhibitory peptide (109) all acting via specific receptors on the β -cell. Inhibitors of insulin secretion include catecholamines (adrenaline and nonadrenaline) which interact with adrenergic receptors on the β -cell membrane (110), and somatostatin which is secreted by δ -cells of the pancreatic islets (111).

Mutations in the insulin gene were first described by Tager et. al. (112) and are known to affect either proinsulin processing or the affinity of the mutant insulins for receptor. All of these mutations have been found to result from single amino acid substitutions producing an altered hormone or prohormone, and are often associated with glucose intolerance and mild diabetes (113,114). These mutations will be discussed later along with an overview of the primary and secondary structures of native insulin. The next section, however, will review the molecular mechanisms of β -cell secretion, i.e., insulin exocytosis.

BIOCHEMISTRY AND ELECTROPHYSIOLOGY OF INSULIN SECRETION

Secretion of insulin from the β -cell is not only an important step in the regulation of glucose homeostasis in healthy individuals, but has also been demonstrated to be inadequate in the diabetic state (115). In fact, in the prediabetic state of type I diabetes, as well as, in various forms of type II diabetes, abnormalities in insulin secretion are an integral component of the pathophysiology (116,117). The β -cell also serves as a model of the secretory process for other cell types having been extensively studied.

Insulin is stored in large dense core vesicles (LDCV) and is released via exocytosis as mentioned previously. This is a multistep process that consists of the transport of the secretory vesicles to the plasma membrane, then docking, priming, and finally fusion of the vesicle with the plasma membrane. It is well known that this process is regulated cooperatively by nutrients, other hormones, and neurotransmitters to cause the electrical depolarization of the β -cell and release of insulin. However, only a small portion of the insulin stored in vesicles in the β -cell is released even under maximum stimulation. This suggests that the systemic insulin levels are therefore regulated by secretion instead of synthesis or storage pools.

The best characterized mechanism of coupling glucose metabolism to insulin secretion resides in the electrical excitability of the β -cell. A large number of ion channels, pumps, and transporters contribute to intracellular calcium concentration, as well as other ions, to form the membrane potential (V_m) of the β -cell of ~ -70 mV when extracellular glucose is ~ 3 mM (see Figure 4). In 1968, Dean and Mathews demonstrated that β -cells were electrically excitable and that glucose controlled this excitability (118,119). They also showed that the action potentials of β -cells were increased by sulfonyleureas. The use of patch clamp techniques allowed the electrical activity of the β -cell to be studied in detail, and elucidated the key role of ATP-sensitive potassium (KATP) channels in the resting membrane potential of the β -cell as well as the importance of these channels in insulin secretion (120-122).

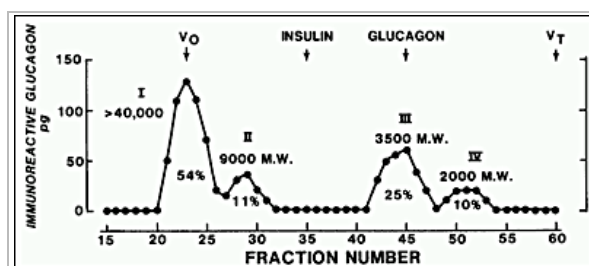


Figure 4. Beta cell ion channels.

KATP channel conductance predominates in the resting β -cell, maintaining the resting V_m near the electrochemical potential (E_K) for K^+ (~ -80 mV). These potassium channels belong to the inward rectifier (K_{ir}) subfamily. They obtain their name from the fact that these channels conduct K^+ current into the cell more readily than to the outside of the cell. KATP channels are, however, weak inward rectifiers because they pass a significant amount of current in the outward direction. At -70 mV, open KATP channels carry a small amount of outward current, which maintains the hyperpolarized resting potential in the β -cells.

A miriade of biochemical and biophysical structure-function studies of recombinant K_{ir} channels have led to a more complete understanding of these channels. The high resolution crystal structure of a bacterial K_{ir} analog, the *Streptomyces lividans* KcSA channel, has been solved (123); and recently, the inner pore of a mammalian K_{ir} has been crystallized and its structure determined (124). The structure revealed that K_{ir} channels consist of four subunits: each subunit folds into the membrane to form two transmembrane domains (M1 and M2) surrounding a pore loop (P). The four P-loops line the central ion-conducting pore with the M1 and M2 subunits providing what appears as outer support (Figure 5).

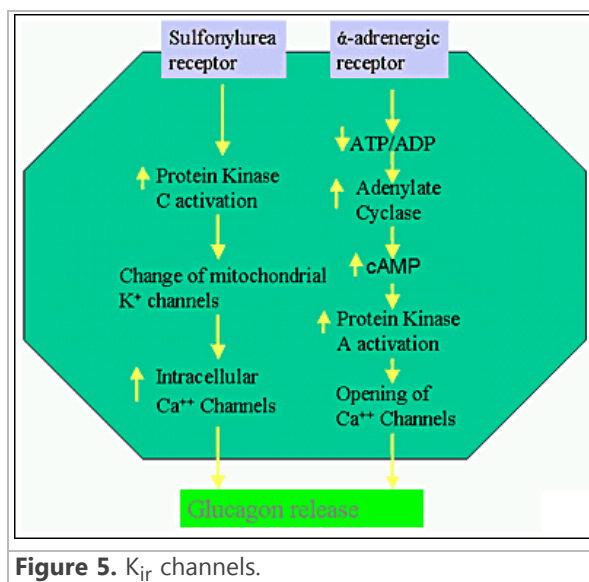


Figure 5. Kir channels.

KATP channels are unique in the inward rectifier family because they require an auxiliary subunit, the sulfonylurea receptor (SUR1), to function. The SUR1 is a member of the ATP binding cassette (ABC) family of membrane proteins, which includes the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel among others (125). KATP channels in the β -cell consists of Kir6.2 subunits surrounded by their accompanying SUR1 subunit (Figure 5).

Elevation of glucose concentration to $>8 - 10$ mM results in the depolarization of the β -cell. Glucose is taken up into the β -cell by the GLUT2 transporter and is metabolized via glycolysis and in the mitochondria to generate ATP. This alters the ATP/ADP ratio, which causes closure of the KATP channel and depolarization of the cell via the decreased K⁺ permeability. ATP inhibits KATP channels and ADP opens them (126,127). Other nucleotides generated by glucose metabolism (Ap3A: diadenosine triphosphate and Ap4A: diadenosine tetraphosphate) have been implicated as second messengers mediating the closure of KATP channels, but their significance is uncertain. Mutations in either the Kir, or SUR1, can result persistent activation with hyperinsulinemia and hypoglycemia in infancy (128). It is also known that some Kir channels in β -cells are activated via G-protein coupled receptors (129-131) which will be reviewed later in this section.

Pioneering studies by Katz, Miledi, and Douglas first established intracellular Ca²⁺ concentrations as the coupling factor between membrane depolarization and vesicular exocytosis (132,133); and this mechanism holds true for β -cells (134). These voltage dependent Ca²⁺ channels (Cav) open upon membrane depolarization caused by Kir channel closure; and it is this Ca²⁺ influx which leads to insulin secretion.

Cav channels are classified as either low voltage threshold (LV: activated at more negative potentials), or high voltage threshold (HV: activated at relatively depolarized potentials). HV channels can be further divided into subclasses: L, N, P, Q, and R (135). Insulin secretion is inhibited by dihydropyridine calcium channel blocking agents, which block L-type Cav. Activators of L-type Cav can stimulate insulin secretion, however, Cav1.3 knockout mice showed perturbed islet function resulting in glucose intolerance and KO mice were smaller than controls, yet maintained glucose-dependent secretion (136). This was secondary either to the upregulation of other Cav1.2 channels, or the existence of other mechanisms of insulin secretion that does not involve voltage gated channels, which will be briefly discussed next.

Several hormones and neurotransmitters regulate insulin secretion in addition to the voltage sensitive pathways. Molecules such as epinephrine, galanin, somatostatin, acetylcholine, and glucagon-like peptide (GLP) all help regulate insulin secretion by binding to their cognate receptors. Cholecystokinin and acetylcholine potentiate insulin secretion via phosphoinositide catabolism with the subsequent mobilization of intracellular calcium. These ligands bind to G-protein coupled receptors that can activate phospholipase-C (PLC). PLC hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) produces inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Two families of Ca²⁺ channels are present on the endoplasmic reticulum: IP₃ receptors and ryanodine receptors (RyR). Both of these are capable of causing the release of Ca²⁺ stored in the ER. DAG concomitantly causes the activation of protein kinase (PKC). Other potentiators of insulin secretion, such as GLP-1, and glucose-dependent insulinotropic polypeptide (GIP), bind to their respective heterotrimeric G-protein coupled receptors to activate adenyl cyclase and increase intracellular cAMP, and subsequently cause the activation of protein kinase A (PKA). Stimulation of either PKC or PKA alters second messenger systems in the β -cell and can chemically modify ion channels to cause a direct influence on insulin secretion. In fact, insulin exocytosis can be induced independently from Ca²⁺ fluxes in in vitro studies by intracellular application of GTP, and the nucleotide analogs GppNHp and GTP γ S.

Specific proteins are also likely involved in the interaction of secretory vesicles with the plasma membrane. Pairing proteins on the vesicle membrane (v-SNARES) have been found to tightly interact with cognate proteins on the target membrane (t-SNARES). Cytosolic cofactors such as N-ethylmaleimide-sensitive factor (NSF) and α/β -SNAP may assist with ATP binding and hydrolysis to cause exocytosis (137).

In addition to voltage sensitive pathways, and heterotrimeric G-protein coupled receptors, the monomeric G-proteins, such as Rab3 (138), may activate second messenger cascades resulting in exocytosis; and multiple Ca^{2+} binding proteins like synaptotagmin (139) may further regulate the event. Nonetheless, the process of insulin secretion is a complex set of events; and research continues to provide new insights on β -cell molecular biology and electrophysiology for the purpose of obtaining a better understanding of insulin action, and its dysregulation that results in diabetes.

INSULIN STRUCTURE

The 3-dimensional structure of insulin has been studied in great detail, and has provided extremely valuable information regarding its function. As previously mentioned, insulin was first crystallized in rhombohedral form in 1926 (36); and almost 10 years later Scott elucidated the importance of zinc, and other divalent cations, in crystallization (140,141). In 1969 the structure of hexameric 2-Zn insulin was determined by Dorothy Hodgkin and her coworkers using X-ray methods (65,142); and was later refined to 1.5 Å (143). Currently there are several crystal forms of insulin that have been solved, all of which display a general similarity to the initial 2-Zn insulin hexamer.

The 2-Zn insulin hexamer (MW~36000) consists of six molecules of insulin (MW~6000) arranged as three dimeric units which possess a three fold symmetry axis (144) (See Figure 6). The dimers (MW~12000) possess a pseudo two-fold symmetry axis which is perpendicular to the three fold axis of rotation. Although each monomer of the dimers has the same peptide backbone structure they are not identical in the arrangement of certain side chains, breaking the perfect two fold symmetry. The most obvious difference is that the side chain of PheB25 is folded in towards the hydrophobic core of its respective monomer in molecule I, and out away from the monomer in molecule II (65). Two Zn^{2+} atoms are aligned with one 8 Å above and one 8 Å below the dimer pseudo two-fold axis of rotation, lying on the three fold symmetry axis of the hexamer. Each ion is octahedrally coordinated by three HisB10 residues and three water molecules.

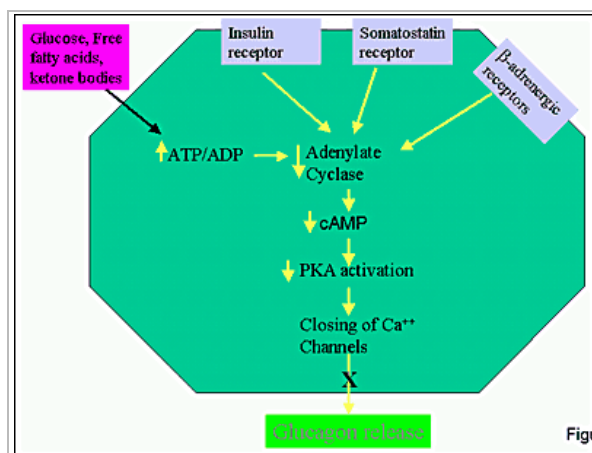


Figure 6. Assembly and disassembly of the 2-Zn insulin hexamer. The A-chain of insulin is illustrated as a thickened black line, and the B-chain as a thinner line. As explained in the text, monomers assemble into dimers as the concentration of protein increases, and into hexamers in the presence of divalent cations such as zinc. The side chains of the six HisB10 residues are shown coordinating with the zinc ions in the hexamer. The pathway is reversed when the hexamer is secreted from the β -cells and diffuses down its concentration gradient into the blood.

The 2-Zn insulin structure has provided a great deal of information about the hydrophobic, solvent exposed, and potential binding surfaces of insulin. Several 2D NMR solution structures of the insulin hexamer (145), dimer (146), and monomer (147,148) have recently been elucidated. Many additional X-ray structures of insulin (148,149), insulin derivatives and insulins of other species, such as the Atlantic hagfish, *Myxine glutinosa* [HisB10 \rightarrow AspB10 substitution preventing Zn-binding and hexamerization] have also been examined (150,150). In most of these instances the insulin, or derivative, maintains an overall tertiary structure that corresponds well with the 2-Zn structure. For this reason the

2-Zn insulin hexamer is often used as the prototypic insulin structure, and this structure was assumed to represent the active conformation of the hormone.

Insulin exists primarily as a monomer at low concentrations ($\sim 10^{-6}$ M) and forms dimers at higher concentrations at neutral pH (152,153). At high concentrations and in the presence of zinc ions insulin aggregates further to form hexameric complexes (65,153,154). Here we shall begin with a discussion of the insulin monomer, which is the active state of the molecule in plasma, and subsequently discuss higher aggregates of insulin.

The Insulin Monomer

As mentioned previously, insulin consists of a (21 residue) A- and (30 residue) B-chain which are disulfide linked, but are illustrated separately in Figures 7a and b, respectively. Insulin maintains a remarkable degree of secondary structure for such a small molecule, as Figure 3 illustrates. The A-chain consists of two helical regions from A1-A8 and from A13-A20 that are nearly antiparallel. The two helices are connected by a stretch of amino acids from A9-A12. Figure 3 illustrates that the N- and C-termini of the A-chain are brought into close proximity by a bend in this extended A chain segment. The B-chain of insulin (Figure 7b) displays an extended chain from B1-B5 and a (nearly antiparallel) central α -helix is preceded by a sharp 1 \rightarrow 4 turn which is allowed by glycine B8. Therefore, the B-chain helix begins with 3:10 contacts made by B7, B8, and unfavorably B9 (143). At B9 the helix hydrogen bonding pattern becomes 1 \rightarrow 5, or α -helical, and is maintained to B19. The B chain continues forming a 1 \rightarrow 4 β -turn with the carbonyl oxygen of B19 hydrogen-bonded to the amide hydrogen of B22, and the carbonyl oxygen of B20 hydrogen-bonded to the amide hydrogen of B23. This β -turn brings the remainder of the B-chain, residues B23-B30, in close proximity and antiparallel to the central B-chain helix. Residues B23-B30 have a β -strand structure with PheB24 and TyrB26 in contact with leucines B11 and B15 of the central B-chain helix. At each end of the B-chain helical-segment, cysteine residues in disulfide linkage to the A-chain (A7-B7 and A20-B19) stabilize the native insulin structure .

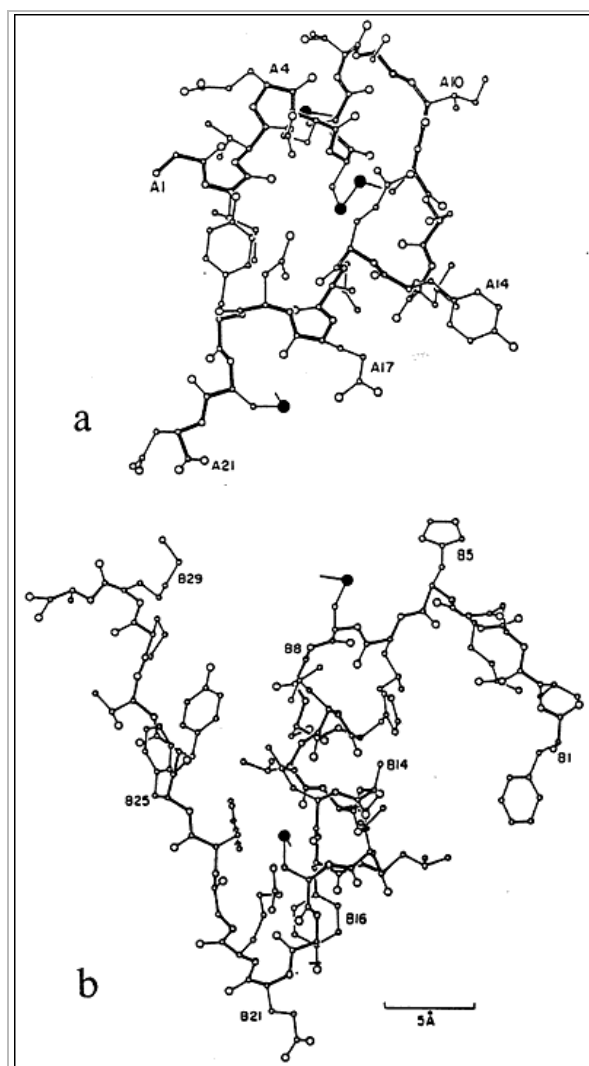


Figure 7. The structures of insulin A- and B-chains. The figure illustrates insulin A-chain (a) and B-chain (b) as determined from the 3-dimensional X-ray analysis of the 2-Zn insulin hexamer. Both chains are viewed perpendicular to the 3-fold symmetry axis of the insulin hexamer (See text).

Joining of the A- and B-chain buries the A6-A11 disulfide bond, leucines A16, B11, and B15 along with isoleucine A2. Along with additional contacts with PheB24, ValA3, IleA13, ValB18, and ValB12 these residues serve to form the hydrophobic core of an insulin monomer, important in maintaining the structure of the molecule. The latter group of residues also make up the surface of the molecule which is partially exposed to solvent in the monomer and is involved in dimer and hexamer stabilization which will be discussed. PheB24, ValB12, and LeuB15 interact to stabilize the β -turn of the B-chain (B20-B23) which allows the β -strand of B23-B30 to fold against the B chain central helix and hydrophobic core of the molecule.

The main chain atoms of PheB25 are also in hydrogen bond contacts with the main chain atoms of TyrA19. Figures 8 illustrate molecules I and II, respectively, which primarily differ in the orientation of PheB25. In molecule I PheB25 is folded against the hydrophobic surface of the molecule while in molecule II PheB25 is displaced outward away from the monomeric core. Molecules I and II also display notable differences in A-chain structure. In Molecule I the A1-A8 helical segment displays both α -helical and β -helical (1 \rightarrow 6 hydrogen bonding) character. In molecule II the same segment of helix is less distorted and is primarily α -helical. The COOH-terminal A-chain helix in both structures is consistent throughout and displays 1 \rightarrow 4 hydrogen bonding and is considered a 310-helix. Molecule I and II of insulin illustrate one general principle, that insulin has a well defined highly organized structure stabilized by specific amino acid backbone and side chain contacts. Accordingly, the disruption of this careful balance of contacts may lead to alterations in the ability of insulin to interact with its receptor (113,114). The removal of residues B26-B30 in despentapeptide insulin (DPI) produces a fully monomeric insulin, incapable of forming dimers (155). This, however, has very little effect on the other residues even though much of the hydrophobic core of the molecule is exposed. These observations, and many others not cited, have demonstrated that the overall tertiary structure of insulin is very stable and, as might be anticipated, is maintained in higher order aggregates.

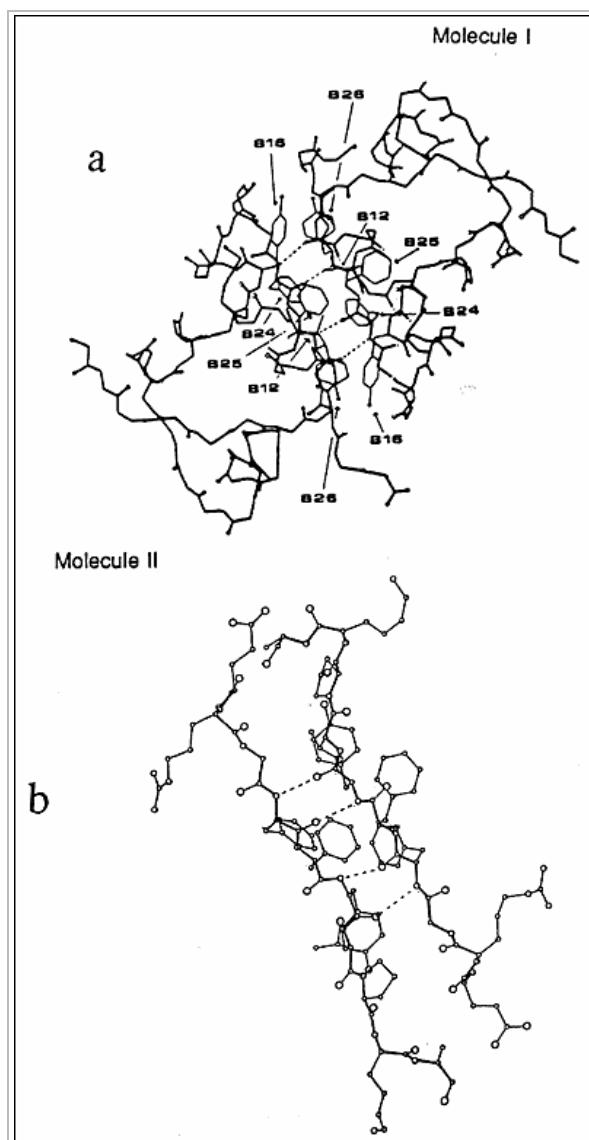


Figure 8. Structural illustration of the monomer-monomer interface in the insulin dimer. The dimer is viewed along the crystallographic 2-fold axis. The side chains of residues ValB12, TyrB16, PheB24,

PheB25, and TyrB26 which form the core of the insulin dimer are illustrated in the figures. Four hydrogen bonds are formed from the main-chain atoms of PheB24 and TyrB26 are illustrated as dotted lines. In the figure (b) is a magnified view of the dimer interface in (a).

The Insulin Dimer

Figure 8a illustrates how molecules I and II interact in the formation of a dimeric complex. The COOH-terminal regions of each B-chain come together to form an antiparallel β -sheet structure which is held together by hydrogen bonding and hydrophobic interactions. Figure 8b illustrates that the dimer interface is a nonpolar core consisting of ten primary residues: ValB12, TyrB16, TyrB26, PheB24, and PheB25 of each monomer in the dimer. These residues are shielded from contact with solvent with the exception of PheB25 which lay on the surface of the dimer. In the monomer, the B-chain β -strand (dimer forming surface) is exposed to solvent; therefore, dimerization occurs via the exclusion of solvent and may contribute to an increase in entropy which favors the aggregation of insulin. It is well known that insulin readily associates to form dimers with a KD of approximately 10^{-5} determined via different techniques (153,156-158).

As mentioned earlier, in 2-Zn insulin the dimeric unit departs from exact 2-fold symmetry; this is due to the different orientation of PheB25 in each monomer. The dimeric unit consists of a molecule I and a molecule II insulin monomer. In molecule I PheB25 is folded back over its respective monomer and interacting with TyrA19; and in molecule II PheB25 is folded away from its own hydrophobic surface over the dimer interface to interact with the adjacent monomer. However, electron density patterns of the 2-Zn insulin structure at 1.5 Å resolution demonstrates that PheB25 of molecule II sometimes assumes a conformation similar to molecular I (143). The actual orientation of PheB25 in solution is likely an intermediate conformation (146,148,159-161).

The Insulin Hexamer

In the presence of Zn^{2+} , insulin dimers associate to form hexameric units coordinated by two zinc ions (insulin $_6Zn^{2+}_2$) (Figure 6). In the hexamer the Zn^{2+} ions are coordinated to the imidazole groups of HisB10 (65) and in a particular instance to HisB5 (162,163). Several hexameric forms of insulin have been crystallized; and among these solved structures 2-Zn insulin remains the prototype. In the 2-Zn insulin hexamer three dimers are related by a 3-fold symmetry axis which is located in the hydrophilic pore at the center of the hexameric unit that connects the two Zn^{2+} ions which lay 8 Å above and below the plane of the hexamer (65). The three-fold symmetry axis is perpendicular to the approximate two-fold symmetry axis of the dimers. As a consequence, contacts between dimers in the hexamer are looser than contacts between monomers in the dimer (144). The vander Waals separations in the hexamer are also greater than those in the dimer. In the 2-Zn insulin hexamer the two centrally located Zn^{2+} ions are on the three fold axis. Each zinc ion is octahedrally coordinated to three HisB10 imidazole ring nitrogens and three water molecules. The entire hexamer is approximately 50 Å in diameter and 35 Å in height (65).

It is unlikely that a substantial amount of hexamer exists in the plasma even though the equilibrium constant for hexamer formation $6(\text{insulin}) + 2(\text{zinc}) = (\text{insulin})_6(\text{Zn}^{2+})_2$ has been estimated to be on the order of 10^{-22} , and 10^{-32} at its highest (65). Therefore, at a typical plasma insulin concentration of 10^{-8} M only about 10^{-33} M hexamer would exist even with the higher $K=10^{-32}$. It is most probable that the monomer is the predominate form in solution with the dimer being the most likely aggregate complex as electrostatic repulsion, and decreased concentration, favor hexamer dissociation after insulin is secreted from the β -cell and enters the systemic circulation.

The 4-Zn insulin rhombohedral crystal form is arranged similarly to the 2-Zn insulin crystal in some aspects. Each dimeric unit consists of one molecule I and one molecule II monomer, and in the hexamer the molecule I trimer has the same octahedral coordination contacts as in the 2-Zn hexamer. Molecule II, however, displays the NH₂-terminus of the B-chain (B1-B8) in a continuous α -helix with B9-B19. This involves a movement of more than 25 Å at B1 (144), and an associated separation of the COOH-terminus (B25-B30) from the A-chain. Three tetrahedral binding sites are created coordinating zinc to two imidazole nitrogen atoms, a chloride ion, and a water molecule, explaining the stoichiometry of 4 zincs per hexamer. However, zinc binding per hexamer is actually 2.67, on average (163).

In the third crystal type, monoclinic 2-Zn insulin, crystals are grown in the presence of zinc and phenol (164,165). In this structure all of the molecules possess a B1-B8 α -helix and separation of the B-chain COOH-terminus from the A-chain. This chain separation allows both molecules I and II to exist in the hexameric unit. Two hydrogen bonds are made with the phenol hydroxyl group to each monomers A6 carbonyl oxygen and A11 amide hydrogen. HisB5 packs each phenol molecule so that tetrahedral coordination now exists between the three imidazole nitrogens of HisB10 and an axial water molecule to generate tetrahedral geometry at each axial zinc ion.

The conformation of insulin in which the NH₂-terminal residues of the B-chain (B1-B8) are extended is referred to as T-state, and when B1-B8 is α -helical is called R-state (Figure 7). This conformational transition from T \rightarrow R has been studied extensively in solutions of hexameric insulin by monitoring changes in the coordination of Co^{2+} (Ni^{2+} and

Co²⁺ also form hexamers) from octahedral to tetrahedral using absorbance spectroscopy (166,167), changes in α -helical content detected using circular dichroism (168,169), or 2D-NMR (170,171,172). All of these studies served to demonstrate that the T6 \rightarrow R6 transition occurs in the presence of phenolic compounds, as well as other cyclic alcohols (167,173). Using circular dichroism, dimers of insulin, along with the monomeric insulin analog [LysB28, ProB29]insulin (168-170) have been demonstrated to be capable of increasing their α -helical content in the presence of cyclohexanol (198). This increase in α -helix was calculated to be consistent with monomeric forms of insulin being capable of assuming an R-like conformation in solution. These studies therefore revisited the question of the potential relevance of the T \rightarrow R transition in insulin receptor binding and activation.

INSULIN STRUCTURE-FUNCTION RELATIONSHIPS

The three dimensional X-ray structure of insulin allowed specific residue positions and side chain orientations to be related to synthetic, as well as, genetic mutants of insulin to obtain a clearer understanding of which residues and positions are necessary for receptor binding (65). Currently, several specific regions of insulin are known to be important in receptor binding. These regions have been determined by the preparation of different insulin analogs bearing amino acid substitutions and deletions, and assaying these analogs for their ability to activate insulin receptor. Several assays have been used to determine the binding potency of insulin analogs such as (a) the in vivo mouse convulsion assay, (b) in vitro receptor binding studies of analogs in competition with radioiodinated insulin, and (c) by the ability of insulin analogs to enhance ¹⁴C-glucose oxidation, or conversion of ³H-glucose into lipids in adipocytes (172). Most of these studies show a strong correlation between receptor binding and biological activity (65,173,174), except for high binding affinity analogs (>120%) which show only 100% activity in vivo probably due to rapid clearance or very low binding analogs which may accumulate at the cell surface and generate a higher than expected activity due to decreased clearance (175).

Three regions in insulin have been of particular concern in attempts to determine how insulin interacts with receptor, because residues in these regions have been found to be evolutionarily conserved. They are the NH₂-terminal A-chain (GlyA1-IleA2-ValA3-GluA4 or AspA4, COOH-terminal A-chain (TyrA19-CysA20-AsnA21), and the COOH-terminal B-chain (GlyB23-PheB24-PheB25-TyrB26) residues. All of these residues are located on or near the surface of insulin and therefore may interact with insulin receptor (176). Several genetic mutants of human insulin have been discovered which have low binding affinity to insulin receptor, display mild glucose intolerance, and cause hyperinsulinemia; all of these mutated insulins display amino acid substitutions in the conserved regions. A LeuB25 for PheB25 substitution (Insulin Chicago), a SerB24 for PheB24 substitution (Insulin Los Angeles), and a LeuA3 for ValA3 substitution (Insulin Wakayama) are mutations which all occur at the gene level and result in insulins with 1-5% of native binding (177-182).

A-Chain Analysis

As previously mentioned, the NH₂-terminal residues of the A-chain (GlyA1-IleA2-ValA3-GluA4 or AspA4) are evolutionarily conserved and have accordingly been investigated for their relevance in ligand receptor interactions. N-acetylation of the A-chain NH₂-terminus results in a reduction of receptor binding to approximately 30%, suggesting the importance of a positively charged free amino group at A1 (173). Deletion of GlyA1 also results in a reduction in receptor binding to 15% of native hormone. These results suggested that GlyA1 is relevant in insulin binding possibly due to the correct positioning of the positively charged NH₂-terminus which forms a salt bridge with the carboxy terminus of the B-chain (65). Disruption of this interaction may distort the structure of insulin enough to perturb receptor binding. Substitution of GlyA1 with L-amino acids (Ala, Val, Leu, Pro, Trp, Lys, or Glu) results in analogs having reduced binding of 2-20%; however, the substitution of GlyA1 with D-amino acids (D-Phe, D-Leu, D-Trp, D-Ala, D-Lys, or D-Glu) results in analogs with full biological activity (183-187). These results suggested that conformation may be of greater importance than a specific side chain, and that the glycine at A1 may actually serve to allow D-conformations (D-like dihedral angles) to be achieved. Mutations disrupting the NH₂-terminal A-chain α -helix, have also demonstrated reduced biological activity coincident with decreased molar ellipticity on circular dichroic analysis (188). The importance of ValA3 has been discussed previously ([Insulin Wakayama-[LeuA3]insulin).

The invariant COOH-terminal A-chain residues (TyrA19-CysA20-AsnA21) have also been studied, as mentioned in the case of the potentially essential TyrA19-IleA2 interaction. Mono- or diiodination of TyrA19 is associated with a reduction in activity (20-50%: Ref. 189) and [TrpA19]insulin displays < 5% activity (190,191). The other tyrosine in the A-chain (TyrA14) does not cause a decrease in receptor binding upon iodination (192,193), and this derivative is therefore used as a radioactive tracer in receptor binding assays (194,195). Treatment of porcine insulin with carboxypeptidase A results in the removal of AsnA21 and AlaB30 (des[AsnA21, AlaB30]insulin) which was found to have 4% of biological activity (196). The determination that AlaB30 is of no significance in receptor binding (197) demonstrated that the low binding affinity of des[AsnA21, AlaB30]insulin is the result of removal of AsnA21. This may be the result of eliminating critical interactions with other residues, such as the A21 amide hydrogen bond to the main chain carbonyl of GlyB23 (65).

CRITICAL ANALYSIS OF INSULIN B-CHAIN DOMAINS

The B-chain of insulin is by far the most studied portion of the molecule, particularly in the COOH-terminal region. As mentioned previously, it is the B-chain NH₂-terminus (B1-B8) that is involved in the T \rightarrow R transition which is accompanied by a concomitant movement of the B-chain COOH-terminal β -strand, demonstrating the conformational

variability of the B-chain (171,198). Accordingly, nucleotide substitutions affecting the transcript region of the human insulin gene which codes for critical B-chain residues produce low binding affinity insulin mutants (113).

Importance of the B-Chain NH₂-terminus

The NH₂-terminal B-chain residues have been studied and data have revealed that the first four residues (PheB1-ValB2-AsnB3-GlnB4) can be deleted with only modest reductions in biological activity (60-70% that of insulin in lowering blood glucose in rabbits) (199,200). Further removal of HisB5 results in an analog, des(B1-B5)insulin, which possesses only 15% activity (200,201). These results suggested that the amino terminal residues of the B-chain are of questionable significance in conferring a high affinity receptor binding state. The lack of a defined orientation for residues B1-B4 in the different T-state crystal structures of insulin (144) has been believed to support this hypothesis; however, some authors speculate that this region of insulin may be of structural/conformational relevance in receptor binding, possibly via the T → R transition which involves the extension of the central B-chain helix to include B1-B8, as well as, other dynamic changes in insulin structure (198).

It has been determined that LeuB6 is critical to insulin binding (202). Deletion of LeuB6 from a parent des(B1-B5)insulin results in a compound with < 1% binding affinity for insulin receptor; and the substitution of LeuB6 for other amino acids (Gly, Ala, and Phe) in full length insulin results in reduced binding [< 0.1%-10%] in the order presented (202). The importance of CysB7 is obvious since it provides a critical disulfide bond linking the A- and B-chain (A7-B7) while GlyB8 and GlyB20 terminate the central B-chain helix at each end.

HisB10 has also been found to be a critical B-chain residue. A genetic mutation results in the substitution of HisB10 to AspB10 producing [AspB10]proinsulin (Proinsulin Providence) which appears to be inefficiently processed to insulin, resulting in an increase in circulating proinsulin [hyperproinsulinemia] (203,204). [AspB10]insulin has been chemically synthesized and surprisingly, has a greatly increased (500%) binding affinity (205). In vivo expression in mice, or in cultured β-cells, has confirmed that [AspB10]proinsulin can be fully processed at normal rates, but a large fraction of unprocessed mutant prohormone is released via an unregulated-constitutive pathway (206,207). Proinsulin processing is also impaired when Arg65 of the C-peptide COOH-terminal dibasic pair (Lys64-Arg65) is mutated to His65 or Leu65 (Proinsulin Boston/Denver/Tokyo and Proinsulin Kyoto, respectively) (208,209). In all these cases the defect lies in the inability of processing enzymes (PC3/PC1 and PC2) to cleave at the mutated cleavage site leading to the secretion of a partially cleaved intermediate form of proinsulin.

Importance of the B-Chain COOH-terminus

The B-chain COOH-terminus is the most widely investigated region of insulin from both a structural, as well as, functional view point. The critical, and evolutionarily conserved, residues GlyB23-PheB24-PheB25-TyrB26 in this region have been previously mentioned and their importance in receptor binding has been demonstrated by [LeuB25]insulin (Insulin Chicago) and [SerB24]insulin (Insulin Los Angeles) which both display a nearly 100-fold decrease in receptor binding (113,114). Deletion analysis has revealed that the COOH-terminal residues TyrB26, ThrB27, ProB28, LysB29, and AlaB30 may be removed, resulting in despentapeptide(B26-B30)insulin which has 20% of the binding potency of insulin (173,210). Interestingly, when the COOH-terminus at B25 is amidated in this truncated analog, despentapeptide(B26-B30)[PheB25-α-carboxamide]insulin, >110% of receptor binding is recovered, demonstrating the deleterious effect of the negatively charged group in this truncated analog (211,212).

Removal of residues B26-B30 results in an analog which lacks critical residues for dimerization, and should result in an insulin analog incapable of forming dimers, ie. monomeric. Circular dichroic studies have demonstrated the inability of despentapeptide(B26-B30)insulin to form dimers, or show spectral changes upon the addition of zinc (211,212), unlike insulin (213). The X-ray crystal structure of despentapeptide(B26-B30)insulin illustrated that the truncated analog is strikingly similar to insulin (214), and this has been confirmed via 2D-NMR studies to also be the case in solution (147,215). Further removal of residues PheB25 and PheB24, deshexapeptide(B25-B30)[PheB24-α-carboxamide]- and desheptapeptide(B23-B30)[GlyB23-α-carboxamide]insulin, results in analogs with 6% and 0.2% binding, respectively; and the subsequent removal of GlyB23 produces desoctapeptide insulin (DOI) with 0.1% binding (216).

Specific Importance of PheB24

The reduced binding as a result of the substitution of PheB24 for SerB24 in Insulin Los Angeles, and for AlaB24, both demonstrate the importance of PheB24 with both analogs having a binding affinity for receptor of < 1%. (217-219). [D-AlaB24]insulin, on the other hand, has ~150% binding affinity (220,221) and [D-PheB24]insulin possesses ~180% binding relative to insulin (222). These results suggest that a D-conformation at position B24 is more favorable than natural L-amino acids, and this appears to be more related to the conformational state conferred by a D-amino acid at position B24 than the specific amino acid side-chain. The requirement for conformational flexibility, in addition to the ability to assume D-orientations at the B-chain COOH-terminus, is supported by the 100-fold reduced (1%) binding of [D-ProB24]insulin (221).

PheB24 is known to contribute two hydrogen bonds critical to dimer formation (65,143), and the substitution of this residue by L- or D-amino acids may be detrimental to the ability of insulin to form dimers. A synthetic insulin analog which has a PheB24 → GlyB24 substitution ([GlyB24]insulin) (221) has 70-80% binding potency and, as is the case with other PheB24 substituted analogs mentioned, also demonstrates a drastically reduced ability to dimerize in

solution. The relatively high binding affinity of this analog has been suspected to be due to the ability of glycine to assume D-like dihedral angles, mimicking the high-potency D-B24 substituents (221). Initially, the 2D-NMR structure of [GlyB24]insulin in 3 M acetic acid revealed that the B-chain COOH-terminus in this analog was completely unfolded away from the remainder of the molecule (223,224); however, subsequent fluorescence studies utilizing tryptophan substituted insulin analogs demonstrated that [GlyB24TrpB25]insulin maintained a near native fold (225). This suggested that [GlyB24]insulin maintained the characteristic insulin-fold, explaining the near normal receptor binding-affinity of this insulin analog (221). Later the 2D-NMR structure of [GlyB24]insulin at neutral pH was solved, and also demonstrated that the native insulin fold is maintained (226).

Specific Importance of PheB25

The relevance of PheB25 has been extensively studied due to the detrimental effect of the genetic PheB25 → LeuB25 mutation in Insulin Chicago (227). Substituting PheB25 with Ala also causes a 10-fold reduction in binding (228). These analogs stress the importance of PheB25 in insulin receptor interactions, while the different orientations of PheB25 (molecules I and II) in the 2-Zn insulin crystal structure (65,143) stress the importance of this residue in conformational variability. It has been shown that although the B25 analogs have low binding potency, unlike PheB24 analogs, their ability to form dimers and zinc coordinated hexamers, as well as, their overall structural characteristics do not appear to be different from those of insulin (228,229). PheB25 is not in the dimer interface like PheB24, and is actually more solvent exposed resting on the surface of insulin adjacent to its hydrophobic core (65,143). Therefore, the substitution of PheB25 does not substantially affect the backbone structure of insulin and can be assumed to interfere directly with side-chain/receptor interactions, or the ability of the COOH-terminus to undergo some conformational change.

Studies have shown that low binding analogs such as [homo-PheB25]-, [LeuB25]-, and [AlaB25]insulin show improvements of up to 100-fold when B26-B30 is removed. These experiments indicate that interaction of the β-aromatic ring of PheB25 with receptor may not be essential for binding and activity, since some truncated analogs which do not possess a β-aromatic ring still bind to the receptor with high affinity. In fact, the conformation of the B-chain COOH-terminus appears to be more important than the specific residues themselves. PheB25 has also been shown to crosslink to insulin receptor demonstrating that this position is adjacent to the α-subunit of insulin receptor upon binding (230). Accordingly, PheB25 may regulate the backbone structure of other COOH-terminal residues in a manner analogous to, but different from, PheB24. In addition, spectroscopic studies have suggested that monomeric insulin undergoes the T → R transition, and that this change is accompanied by a concomitant alteration in the orientation of the aromatic side-chain at PheB25 (See model in Figure 9).

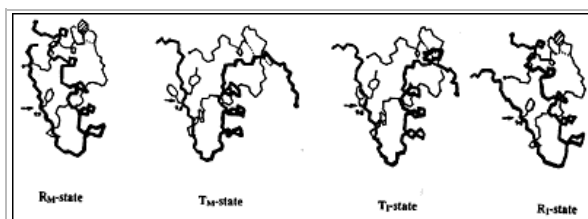


Figure 9. Model of the different conformational states of metal coordinated (hexameric), and monomeric, insulin. The thick line represents the B-chain, the thinner line represents the A-chain of an insulin monomer, and the amino terminus of each chain is indicated by a ball. The arrow in each illustration gives the location of PheB25, cyclohexanol is represented as a striped hexagon, and hydrogen bonds are represented as dotted lines. It can be seen that the major difference between the metal coordinated TM and RM-state is the reorganization of hydrogen bonding at the B-chain NH₂-terminus, allowing two additional turns of helix, and alteration of PheB25 in the RM-state. The TI and RI-state, which are independent of metal ion coordination, differ in a similar manner, however, the β-sheet region of the B-chain COOH-terminus in the RI-state is not as well defined as in the RM-state.

Nearly thirty-five years after the structure of insulin was first solved, it is still unknown how insulin binds to its receptor. Several investigators have demonstrated that N-terminal A-chain residues are important in insulin receptor interactions, as are C-terminal B-chain residues. However, the cumulative data suggest that the overall

structure/conformation, or the ability to undergo conformational changes, appears to be most important factor in high affinity insulin binding. Given the small size of insulin, its ability to assume multiple defined conformations is nothing less than impressive. Accordingly, these conformational changes will possibly prove to occur as a distinct sequence of events, orchestrating the high affinity binding of insulin to the insulin-receptor.

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