A New Interpretation of Structure-Function Relationships in Insulin-Receptor Interactions

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Summary. Arguments are presented for a unified theory of insulin binding which differs from presently accepted viewpoints: (1) Insulin receptors are bivalent with some motional freedom between binding units; (2) following ligand formation a conformational change occurs which leads to a restriction of receptor flexibility; (3) when both binding units are occupied, they can approach sufficiently closely with an antiparallel symmetry to permit dimer formation-related interaction of receptor-bound insulin molecules; (4) this event leads to enhanced dissociation (negative cooperativity) with kinetic

features identical to the model of de Meyts; (5) the binding surface of insulin cannot include residues involved in dimer formation. If this is so, then the accepted receptor binding surface of insulin, which includes the residues involved in dimer formation, is incorrect. Arguments are brought suggesting that strongly conserved hydrophilic residues of the A chain may be involved.

Key words: Insulin structure, insulin receptor, negative cooperativity, insulin binding, insulin association.

The determination of the three-dimensional structure of 2-Zn⁺⁺ insulin crystals in 1969 was an enormous stimulus for the investigation of structure-function relationships in insulin. The geometrical relationships of the amino acid residues of the sequence determined by Sanger et al. 14 years previously were made evident, and the results of preliminary chemical modifications could be interpreted [1]. Since that time literally hundreds of insulin analogues have been synthesized and their properties studied in greater or lesser detail. Although we cannot claim to have a full understanding of the molecule as yet, an enormous body of information is available [2]. In this article I will discuss how insulin interacts with its receptor. Subsequent events, such as the induction of biological responses, are insulin-independent (the complete range of insulin activities can be elucidated with anti-receptor antibodies [3]) and will not be treated.

Insulin Structure

A conventional representation of insulin is shown in Figure 1a, and a highly schematized representation in Figure 1b. The A chain consists of two helical segments (A2–8 and A13–20) linked by a turn sequence. The B chain starts with a β -strand-like structure (B1–6), runs

into a long helical stretch (B9–19), then into a turn, and finally into a short strand of β -structure (B24–29). The two chains are linked by disulphide bonds and stabilized by tight packing of hydrophobic side-chains at the interfaces of the structural elements. The stability of the structure is indicated by the low tendency to irreversible denaturation and by the relatively high yields of insulin obtained on recombination of the separated chains.

There is some spectroscopic evidence to support the assumption that the structure in solution and in the crystal are largely similar [4]. It is uncertain whether this is the same structure as is bound by the receptor. Insulins bearing a cross-link between the A1 and B29 residues have structural properties very similar to unmodified insulin [5], but binding affinities and potencies of the order of only 5–10% [6]. This would seem to imply that a conformational adjustment of the binding insulin is necessary for the full expression of a biological response but there is no information to suggest what this might be.

In the crystal, 2-Zn⁺⁺-insulin is hexameric, in the form of three symmetrically related dimers [1]. The dimer which seems most likely to correspond to that observed in solution is a nearly symmetrical antiparallel association of monomers interacting about their B(24–29) β -strands and adjacent hydrophobic side-chains of the B(9–19) helix. Some asymmetry is evident

Fig. 1 a and b. **a** A detailed representation of the insulin structure (adapted from ref. [1]). **b** A schematic representation of the structural features of the insulin molecule

Fig. 2. A comparison between the residues involved in insulin dimer formation [1], the postulated receptor binding surface [8] and the site responsible for negative cooperativity [9]. Only in the case of the dimer-forming interaction is it possible to define which functions of a given residue are involved (-NH-, -CO-, or side-chain)

Fig. 3. Dissociation of ^{125}I -insulin from IM-9 lymphocytes (adapted from ref. [10]). The upper curve is the dissociation of tracer after dilution into insulin-free medium; the lower curve is the dissociation after dilution into an insulin-containing medium. The difference between the two curves is also shown (-----)

in the dimer, particularly with the B25 phenyl groups [7], suggesting that these side-chains are either unimportant for dimer formation, or only important in guiding the monomers into the dimer state.

The dimer-forming interface features heavily in discussions of structure-function relationships. Figure 2

shows the correspondence between the residues involved in this interface and the residues postulated as responsible for receptor binding [8] and for the modulation of receptor affinity (negative cooperativity) [9].

I will now examine the evidence for this surprising correspondence and present arguments which suggest (1) that negative cooperativity is due to a dimer formation-related interaction between receptor-bound insulin monomers, (2) that the putative receptor-binding region cannot be correct.

Insulin Binding

De Meyts et al. have presented substantial evidence which demonstrates that the affinity of the insulin receptor pool declines with increasing occupancy, an effect described as negative cooperativity [10]. This model explains the curvilinearity of the Scatchard representation of insulin binding and predicts that the loss of affinity is due to an increasing dissociation rate. This has been elegantly demonstrated in experiments which measure the rates of dissociation of bound insulin tracer from a receptor pool on dilution into media free from, or containing, insulin (Fig.3).

When the difference between the amounts dissociated are plotted as a function of time, one observes the accelerated dissociation occurs for only a short time, and levels out at a relatively constant value. There is therefore not merely a change in the 'off-rate' rate of the receptor which occurs with increasing occupancy, but also an isomerization of the receptor to a state in which accelerated dissociation does not occur. One can only speculate as to what this may mean: perhaps it is related to the triggering of post-receptor events.

Fig. 4. An object with twofold rotational symmetry. Note that the top surfaces ($\rightarrow$) are antiparallel to each other

Arguments have been presented to show that the increased dissociation is not due to insulin-insulin interactions [10]. These arguments are: (1) there are insulins which do not form dimers but are cooperative (e.g. guinea pig insulin), (2) there are insulins which do form dimers but are not cooperative (e.g. des-Ala^{B30}, Asn^{A21}-insulin, DAA-insulin; [Leu^{B25}]-insulin).

There is no evidence that hystricomorph insulins form dimers in solution [11]. Nevertheless there is the potential to form dimers, due to the conservation of residues important in dimer formation (Table 1). If it is assumed that the receptor is bivalent, it implies that the two binding sites are arranged with an antiparallel symmetry to each other (Fig. 4). It is easy to imagine that constraining one molecule of iodinated insulin (the tracer) in an attitude and proximity which favours the approach to dimer formation with another molecule of 'non-dimer forming' insulin will enormously increase the probability of interaction. In solution there exists an equilibrium, $\text{Monomer} + \text{Monomer} \xrightleftharpoons[k_{\text{diss}}]{k_{\text{ass}}} \text{Dimer}$, in which the observation of dimer formation depends mainly on k_{diss} ; at the receptor the limiting rate for interaction is k_{ass} .

DAA-insulin is a non-cooperative analogue [12]; it has a linear Scatchard plot and does not enhance the rate of dissociation of bound tracer. On the contrary, it can prevent the accelerated dissociation seen on diluting into insulin-containing media [12]. It has been stated that this analogue forms a dimer with itself with 1% the avidity of insulin. This is based on sedimentation equilibrium studies [13] which showed that the data were best fitted by a scheme of association for insulin 1-mer $\xrightleftharpoons{K_{12}}$ 2-mer $\xrightleftharpoons{K_{24}}$ 4-mer $\xrightleftharpoons{K_{46}}$ 6-mer ($K_{12} = 220 \text{ M}^{-1}$, $K_{24} = 0.036 \text{ M}^{-1}$, $K_{46} = 0.22 \text{ M}^{-1}$); for DAA-insulin however the best scheme was found to be 1-mer $\xrightleftharpoons{K_{12}}$ 2-mer $\xrightleftharpoons{K_{23}}$ 3-mer $\xrightleftharpoons{K_{34}}$ 4-mer ($K_{12} = 1.74 \text{ M}^{-1}$, $K_{23} = 1.11 \text{ M}^{-1}$, $K_{34} = 0.03 \text{ M}^{-1}$).

For insulin, K_{12} is much greater than K_{24} , but for DAA-insulin, K_{12} is very similar to K_{23} . These results would therefore suggest that no specific dimer formation of DAA-insulin occurs, only a non-specific aggregation.

Table 1. Comparison of residues intimately involved in monomer-monomer contacts in bovine and hystricomorph insulins

Insulin (species)	B12	B16	B24	B25	B26
Bovine	VAL	TYR	PHE	PHE	TYR
Guinea pig	VAL	TYR	PHE	PHE	TYR
Chinchilla	VAL	TYR	PHE	PHE	TYR
Porcupine	VAL	TYR	PHE	PHE	TYR
Casiragua	VAL	TYR	PHE	TYR	ARG
Coypu	VAL	TYR	PHE	TYR	ARG

Table 2. Correlation between the potential for interaction of some insulin analogues at an intact B25-A21 surface and their ability to induce negative cooperativity

Analogue	Potential interaction	Cooperativity
Beef insulin	yes	yes
Guinea pig insulin	yes	yes
DAA-insulin	no	no
[Leu ^{B25}] insulin	no	no
DOP-insulin	no	no

[Leu^{B25}]-insulin forms dimers normally [14], but is hardly cooperative [15]. However, as has been mentioned above, residue B25 has an unusual symmetry in the dimer, and it may well be that the approach to dimer formation necessary for this model of cooperativity is more dependent on this side-chain than is the final state of dimer formation. The neighbouring residue Asn^{A21} is also not involved directly in dimer formation per se [17] but its loss seems to prevent dimer formation altogether. Distortions of structure in the region of B25 and A21 are therefore not only important for the approach to dimer formation, but also for the proper maintenance of the structure of the binding interface (both analogues have very low potencies).

A Model for Insulin Interactions

There seems to be a correlation between the potential of receptor-bound insulin molecules to interact through a region about residues B25 and A21, and their ability to induce cooperativity (Table 2). In Figure 5, a model is presented which seeks to incorporate these data [16]. The features are: (1) the receptor is bivalent with antiparallel symmetry between binding units; it can flex so that two bound insulin molecules can interact, thereby enhancing the dissociation rate of one, if not both, ligands; (2) the receptor isomerizes rapidly to an inflexible state when both binding units are occupied; this state does not allow interaction between the ligands to occur.

The 'flexible' receptor state allows ligand interaction to occur, but does not force it to; the extent of interaction will be determined by factors such as the structure in the B25-A21 region and the fluidity of the environment surrounding the receptor.

Fig. 5. The bivalent receptor has two independent binding units which possess dissociation constants K_{d1} , K_{d2} for tracer and insulin analogue respectively. The binding units have an antiparallel symmetry and can approach sufficiently closely that an interaction related to dimer formation can occur at a rate k_{ass} . This leads to an enhanced rate of dissociation of at least one of the partners, and hence negative cooperativity. Insulin dimers can also bind to one unit but are non-cooperative because their dimer forming surfaces are already occupied. The receptor isomerizes rapidly (k_{isom}) to a non-cooperative state with a half-time of 1-2 min at 15 °C in IM-9 lymphocytes; it may revert to its original condition at a rate k_{isom}^- to permit dissociation, or dissociation may occur directly from the non-cooperative state

Fig. 6. Dependence of cooperative effect on insulin concentration (adapted from ref. [12]). The increase in rate of dissociation of bound iodinated porcine insulin (as percentage of the maximal rate of dissociation) is plotted as a function of the concentration of porcine insulin (●—●) and of guinea pig insulin (▲—▲) in the diluting medium. The calculated curve for the percentage of porcine insulin present as dimers for the given concentrations is also shown (-----)

When the extent of the cooperative effect is plotted as a function of insulin concentration [10], three important features are defined (Fig. 6). The half-maximal effect is proportional to the binding affinity (K_d) of most insulins studied and the effect is maximal at concentrations which give approximately 100% receptor occupancy at equilibrium, which is very rapidly achieved at high insulin concentrations. (Some disproportionality between the equilibrium occupancy and the cooperative effect would be expected however when the rate of equilibration is comparable to the isomerization rate of the receptor to the 'inflexible' state.) Further, there is a fall-off in effect at high concentrations of insulins which dimerize in solution.

This latter feature has been ascribed to the equipotent binding of non-cooperative dimers and cooperative monomers [4, 9, 10]. The curves describing the increase in proportion of insulin dimer in solution and the decrease from maximum cooperative effect are superimposable. If the dimer binds as well as the monomer,

then the dimer formation interface cannot be involved in receptor interaction.

Support for this comes from the retention of receptor affinity and biological activity by des-octapeptide (B23-30) insulin (DOP-insulin), although only at a level of 0.1% [17]. Thus, residues B24, B25 and B26 are not as crucial for receptor interaction and biological activity as they are for dimer formation and the maintenance of an intact insulin structure. DOP-insulin cannot form dimers, has a highly perturbed structure [13], and is non-cooperative [9].

One important observation speaks against this model [12]. It has been shown that DAA-insulin cannot accelerate the dissociation of iodinated insulin, but that insulin can accelerate the dissociation of iodinated DAA-insulin. If cooperativity results from a ligand-ligand interaction, one would expect that insulin should not accelerate the dissociation of iodinated DAA-insulin. However, binding data indicate that the iodinated DAA-insulin used in these experiments has a tenfold

Table 3. The residues proposed to be in the receptor binding surface of insulin with sequence variations

A1	A4	A5	A15	A17	A18	A19	A21
GLY	GLU	GLN	GLN	GLU	ASN	TYR	ASN
	Asp ^a		Asp ^b	Gln ^c	Ser ^d		

^a Asp^{A4} found in rat, hystricomorph and cod insulin; ^b Asp^{A15} Gln^{A17} is found instead of the sequence Gln^{A15} Glu^{A17} in fish; ^c Gln^{A17} is found in guinea pig and porcupine; ^d Ser^{A18} is found in hystricomorphs and toad fish

higher affinity, and is therefore more insulin-like than non-iodinated DAA-insulin. It seems possible, therefore, that this is the first reported example of the reconstitution of the insulin structure by introducing a beneficial modification after a deleterious modification. In order to make a proper evaluation of the experimental result, a more appropriate and homogenous tracer DAA-insulin will have to be synthesized.

Conclusion

If the dimer-forming surface is excluded, which insulin surface is involved in binding? The A chain helix surfaces seem to be good candidates. They are strongly conserved (Table 3), which suggests that they may be constrained by a functional role. Such modifications to these surfaces as are known (these are regrettably few due to the technical difficulties in introducing them) lead to large alterations in biological potency [16]. These surfaces would seem to merit more investigation and have indeed been recently reassessed from a structuralist's point of view [5]. It is obvious that we still have much to learn before we can understand properly the insulin molecule.

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