*Biochem. J.* (1983) **216**, 687–694
*Printed in Great Britain*



# Evidence concerning the mechanism of insulin–receptor interaction and the structure of the insulin receptor from biological properties of covalently linked insulin dimers

Michele A. TATNELL,*§ Richard H. JONES,* Kevan P. WILLEY,† Achim SCHÜTTLER‡ and Dietrich BRANDENBURG‡

**Department of Medicine, St. Thomas' Hospital Medical School, London S.E.1, U.K., †Institut für Hormon-und Fortpflanzungsstorungen, Hamburg, Federal Republic of Germany, and ‡Deutsches Wollforschungsinstitut, Aachen, Federal Republic of Germany*

(*Received 12 July 1983/Accepted 25 August 1983*)

Covalently linked insulin dimers have been prepared by cross-linking two insulin monomers with a flexible suberoyl chain at either the B1 phenylalanine or the B29 lysine residue. Binding potencies of dimers determined by inhibition of binding of $^{125}$I-insulin to isolated rat liver plasma membranes or adipocytes were 2.5–7-fold greater than their abilities to stimulate lipogenesis in adipocytes. Rates of liver plasma-membrane-associated degradation of labelled insulin and dimers, measured by gel filtration, were similar at 37°C. Binding and lipogenesis potencies of dimers prepared by substitution of each monomeric half of an asymmetrical dimer with desoctapeptide insulin, an almost inactive derivative, implicated the B1-cross-linked monomeric half as predominantly interacting with the insulin receptor. These results suggest that (1) dimers bind univalently to a bivalent insulin-receptor complex, in which the two individual binding subunits are arranged with anti-parallel symmetry and (2) the mechanism by which insulin binds and initiates its biological responses requires a conformational change within the insulin-receptor complex and/or in the insulin molecule for full biological expression.

The use of insulins of various animal species and many semisynthetic derivatives has been invaluable in identifying and characterizing several properties of the insulin molecule and its interaction with the receptor. Through such studies it has been established that the predominantly hydrophobic *C*-terminus of the B chain plays a key role in the binding event. This 'binding' region has been defined as consisting primarily of A1, A5, A19, A21, B12, B16 and B22–26 residues (Peking Insulin Structure Research Group, 1974; Pullen *et al.*, 1976). Hydrophobicity has been proposed as the major driving and stabilizing force, whereas van der Waals' and polar interactions determine the recognition between two proteins, since they require complimentarity of the two surfaces involved (Sasaki *et al.*, 1975; Waelbroeck *et al.*, 1979).

C.d. studies of modified insulins provided strong evidence that the tertiary conformation of the molecule was vital for its full expression (Peking Insulin Structure Research Group, 1974; Blundell *et al.*, 1972). It is difficult to extrapolate from the known tertiary structure of the insulin molecule in the crystalline form to its conformation in solution or at the receptor site. However, the different spatial arrangements of certain residues found in the two insulin molecules of a natural dimer in the different crystalline forms of insulin, primarily at the *N*-termini of both A and B chains and at residue B25 (Blundell *et al.*, 1972; Peking Insulin Structure Research Group, 1974; Cutfield *et al.*, 1981), indicate some degree of molecular flexibility. It is

Abbreviations used: LPM, liver plasma membranes; DOP, desoctapeptide insulin; B1-B'29 D, [$N^{B1}$,$N^{B'29}$-suberoyl]insulin dimer; B1-B'1 D, [$N^{B1}$,$N^{B'1}$-suberoyl]-insulin dimer; B29-B'29 D, [$N^{B29}$,$N^{B'29}$-suberoyl]insulin dimer; B29-B'1 DOP, des-B23–30-insulin–[$N^{B1}$,$N^{B'29}$-suberoyl]insulin dimer; B1-B'1 DOP, des-B23–30-insulin–[$N^{B1}$,$N^{B'1}$-suberoyl]insulin dimer; I-I dimer, insulin–insulin dimer; DOP-dimer, insulin–desoctapeptide insulin dimer; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

§ Present address: Department of Medicine, Auckland Hospital, Park Road, Auckland, New Zealand.

possible that this flexibility is also important during interaction with the receptor.

A reduction in biological effects of an insulin analogue has usually been directly attributed to a reduction in its affinity for the insulin receptor (Freychet *et al.*, 1974; Gliemann & Gammeltoft, 1974). A few exceptions among monomeric insulin derivatives have been reported, although no satisfactory explanation has yet been found for their lower biological responses (by 3–5-fold) compared with their binding affinities (Cutfield *et al.*, 1979; Burke *et al.*, 1980; Horuk *et al.*, 1980; Rösen *et al.*, 1980).

A detailed study of covalently linked insulin dimers was undertaken, because their unusual biological properties might help to unravel the mechanism(s) involved in the insulin–receptor interaction and initiation of biological responses. In the present paper we describe their binding and lipogenic potencies and their degradation. Substitution of each monomeric half of an asymmetrical dimer with the almost inactive DOP allowed us to determine the separate functional properties of each monomeric half of an I-I dimer. This enables us to offer explanations of some of the characteristics of the dimers themselves, and to propose some possible structural properties of the insulin receptor. A preliminary report of these data was presented at the First International Symposium on Insulin in Aachen, Germany (Willey *et al.*, 1980).

## Materials and methods

Highly purified desamido-free bovine insulin was a gift from Dr. D. Saunders, Deutsches Wollforschungsinstitut, Aachen, Germany. Covalently linked insulin dimers were prepared by cross-linking the amino groups at $Phe^{B1}$ and/or $Lys^{B29}$ with an aliphatic bridge containing eight carbon atoms (Schüttler & Brandenburg, 1982). The three resulting I-I dimers are B1-B'29 D, B1-B'1 D and B29-B'29 D. The two DOP-dimers, B29-B'1 DOP and B1-B'1 DOP, were prepared by methods directly analogous to those used for asymmetrical dimers.

Buffers, each containing 10g of bovine serum albumin/litre (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.), were (1) Krebs–Ringer phosphate (127mM-NaCl, 7mM-KCl, 2.6mM-$MgSO_4$, 22mM-$Na_2HPO_4$, 95mM-HCl, pH 7.8) for LPM binding experiments, (2) Krebs–Ringer bicarbonate (120mM-NaCl, 4.8mM-KCl, 1.2mM-$KH_2PO_4$, 2.5mM-$MgSO_4$, 26mM-$NaHCO_3$, 1.3mM-$CaCl_2$,-$H_2O$, 0.55mM-D-glucose, pH 7.4) for adipocyte lipogenesis assays, (3) Krebs–Ringer/Hepes (the same as the bicarbonate buffer, except that 25mM-Hepes was substituted for $NaHCO_3$) for adipocyte binding assays and (4) 50mM-phosphate buffer (8.9mM-$KH_2PO_4$, 41.4mM-$K_2HPO_4$, pH 7.4) as the wash buffer for the filter separation in LPM binding experiments. This last buffer, with 1g of bovine serum albumin/litre, was used to elute the gel-filtration columns. All radioisotopes were purchased from Amersham International, Amersham, Bucks., U.K. All chemicals were analytical grade.

### *Iodination of ligands*

Insulin and dimers were radiolabelled by the chloramine-T method of Greenwood *et al.* (1963), modified as described previously (Tatnell & Jones, 1981). The iodination mixture was purified on a Sephadex G-50 (fine) column, eluted with 50mM-phosphate buffer. Fractions (0.8ml) were collected and counted for radioactivity. Three main peaks were resolved: high-molecular-weight material in the void volume (peak I); a 12000Da peak with dimers (peak IIa) or a 6000Da peak with insulin (peak IIb); and low-molecular-weight products (peak III). The proportion of intact ligand in peak IIa or IIb was estimated by precipitation with trichloroacetic acid, and was always greater than 90% for labelled insulin and dimers used in binding assays. The specific radioactivities, measured by radioimmunoassay, were $155.16 \pm 18.08$Ci/g for insulin ($n = 17$), $92.13 \pm 9.30$Ci/g for B1-B'29 D ($n = 9$) and $82.41 \pm 13.69$Ci/g for B1-B'1 D ($n = 6$).

### *Adipocytes and liver plasma membranes: preparation and bioassays*

Isolated adipocytes were prepared by digestion of epididymal fat-tissue of 100–120g Corworth–Sprague–Europe rats with collagenase [*C. histolyticum*; Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.] by a modification (Gliemann, 1967) of the method of Rodbell (1964). LPM were isolated from 250g male rats as described by Wisher & Evans (1975). The purified LPM were primarily blood sinusoidal in origin and possessed low insulin-degrading activity (Wisher *et al.*, 1977). Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Binding experiments with isolated adipocytes and LPM were as described in the legend to Table 1. Separation of free radioactivity in the medium from cell- or membrane-bound radioactivity was by oil flotation (Gliemann *et al.*, 1972) for adipocytes and by a modification (Tatnell & Jones, 1981) of the glass-fibre filter method of Cuatrecasas (1971) for LPM. Insulin- or dimer-stimulated lipogenesis was determined in isolated adipocytes by measuring the conversion of D-[3-$^3$H]glucose into toluene-extractable lipids (Moody *et al.*, 1974) for 90min at 37°C. Under these various assay conditions, the mean degradation of $^{125}$I-insulin, assessed by precipitation with trichloroacetic acid, was $6.92 \pm 1.22\%$ ($n = 5$) and

7.78 ± 1.20% ($n = 8$) in binding experiments with adipocytes and LPM respectively, and 1.62% ($n = 2$) in lipogenesis assays.

Degradation of $^{125}$I-insulin, $^{125}$I-B1-B'29 D and $^{125}$I-B1-B'1 D was measured by gel filtration of bound and unbound radioactivity after incubation with LPM as described in the legend to Fig. 2. The nature of the labelled bound material was determined after dissociation from LPM with 0.01 M-HCl, as described by Posner *et al.* (1978). Although maximal dissociation (89.75 ± 0.85%) of $^{125}$I-insulin occurred within 10 min of incubation with HCl, the degree of dissociation of dimers was significantly less than that of insulin [57.67 ± 4.18% for B1-B'29 D ($P < 0.001$) and 71.67 ± 6.17% for B1-B'1 D ($P < 0.05$)]. With dimers a 60 min period of incubation was used in an attempt to dissociate more of the radioactivity, although no more dissociation occurred after about 20 min.

Controls with labelled ligand in the absence of LPM were chromatographed after either no incubation or incubation in (*a*) buffer for 30 min at 37°C or (*b*) 0.01 M-HCl for 60 min. The elution profiles of all samples and controls showed three major peaks, as described in the iodination procedure. Percentage membrane-associated degradation was calculated as described in the legend to Table 2.

### *Analysis of data*

Statistical tests for determining the level of significance of difference between two sets of data were by paired or unpaired Student's *t* tests, and between more than two sets of data by one-way analysis of variance. Two or more logarithmic dose–response curves from bioassays were compared by a parallel-line bioassay analysis (Finney, 1964) using a program written by M. D. Baron for a Z80-A based microcomputer. This program used the data on the linear portion of the curves to determine validity of the assay, potencies of test samples and 95% confidence limits of the test-sample potencies relative to the standard. Variance values throughout the text are always S.E.M.

## Results

### *Binding and lipogenesis potencies*

Inhibition of binding of $^{125}$I-insulin by insulin and dimers was studied in isolated LPM and adipocytes.

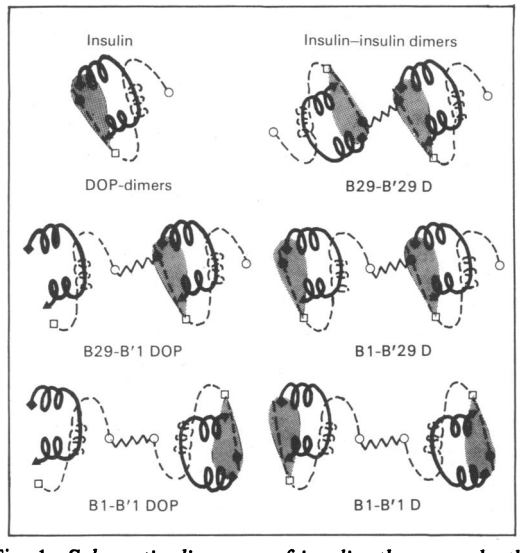


Fig. 1. *Schematic diagrams of insulin, three covalently-linked insulin–insulin dimers and two insulin–desoctapeptide insulin dimers*

A chain (thick continuous line), B chain (broken line) and the putative receptor binding region (shaded area) are shown, as are A1 (♦), A21 (▲), B1 (○), B22 (□) and B29 residues (●). Monomeric halves of each dimer are rotated such that the 'binding' regions are in the same plane.

Table 1. *Binding and lipogenesis potencies of dimers*

In equilibrium binding experiments 100–200 pM-$^{125}$I-insulin and various concentrations of insulin (0.1–1000 nM) and dimers (0.05–500 nM) were incubated with LPM (200–400 μg/ml) for 45 min at 37°C or with isolated adipocytes [(1–3) × $10^5$ cells/ml] for 60 min at 30°C. Cell- or membrane-bound radioactivity was separated from radioactivity in the medium as described in the Materials and methods section. Insulin (8.7–870 pM)- or dimer (34–8800 pM)-stimulated lipogenesis was measured in isolated adipocytes [(1–3) × $10^4$ cells/ml] for 90 min at 37°C. The logarithmic dose–response curves of insulin and the dimers in these experiments were analysed by the parallel-line bioassay method of Finney (1964) to determine the binding and lipogenic potencies of the dimers. Potencies are on an equimolar basis with insulin and are means ± S.E.M. of the numbers of experiments shown in parentheses.

| | Binding potency (%) | | Lipogenesis potency (%) |
|---|---|---|---|
| Ligand | LPM | Adipocytes | |
| B1-B'29 D | 201.98 ± 31.17 (4) | 169.72 (2) | 79.01 ± 4.37 (7) |
| B1-B'1 D | 199.12 ± 23.78 (4) | 157.26 (2) | 28.82 ± 2.48 (8) |
| B29-B'29 D | 74.05 ± 18.38 (3) | 79.74 ± 13.86 (3) | 13.46 ± 0.64 (5) |
| B29-B'1 DOP | 13.35 (2) | | 7.74 ± 0.09 (3) |
| B1-B'1 DOP | 93.46 (2) | | 20.86 ± 0.33 (3) |

Binding potencies from preliminary studies with LPM and hepatocytes involved incubation for 30 min at 30°C (Willey *et al.*, 1978; Schlütter *et al.*, 1980) and are subject to error, owing to failure to attain a steady-state of binding. Binding potencies of dimers, shown in Table 1, were determined at a true steady-state of binding, which was found to be 45 min of incubation at 37°C for LPM or 60 min at 30°C for adipocytes (except that B1-B'-1 D may require even longer incubation to achieve a steady-state in adipocytes). All assays showed parallel binding curves with the exception of those with B1-B'29 D, where 33% of the assays with LPM and 50% with adipocytes had significantly non-parallel binding curves relative to those of insulin.

Results of lipogenesis assays with adipocytes are shown in Table 1. All dose–response curves of insulin and dimers were parallel and attained a similar maximal response. The lipogenic potencies of all dimers were lower than expected from their binding potencies. Binding potencies of B1-B'29 D and B1-B'1 D were twice that of insulin on a molar

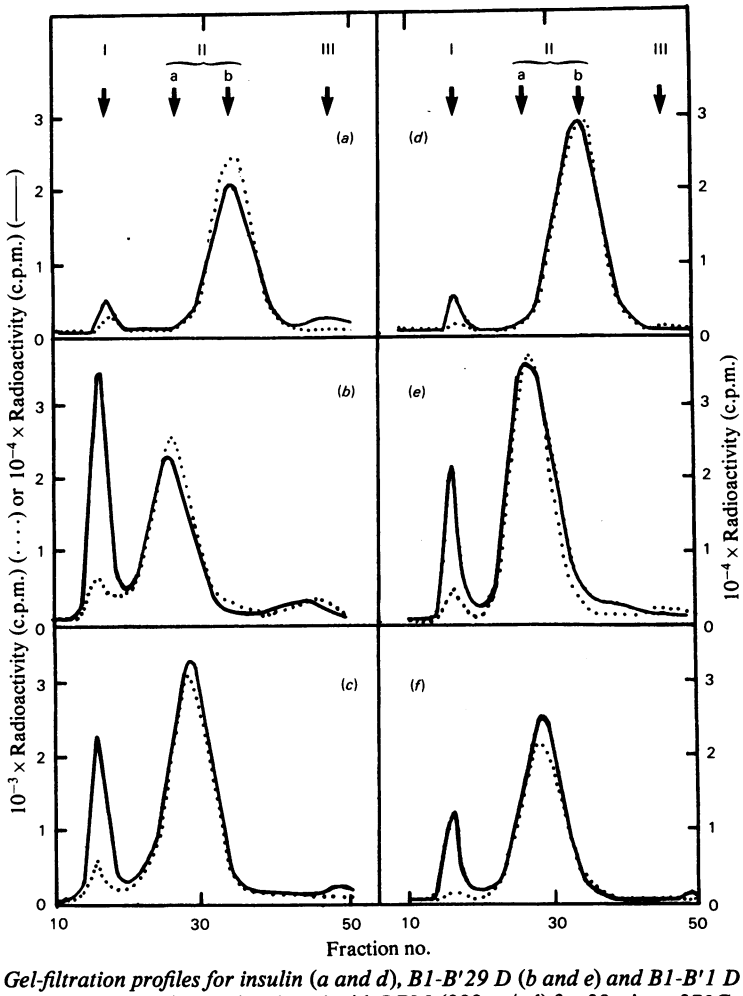


Fig. 2. *Gel-filtration profiles for insulin (a and d), B1-B'29 D (b and e) and B1-B'1 D (c and f)*

(*a–c*), Labelled ligands (100–200 pM) were incubated with LPM (300 μg/ml) for 30 min at 37°C. Sample (500 μl) was centrifuged in a Beckman Microfuge for 10 min at 4°C. Supernatant was removed and chromatographed on Sephadex G-50 (fine) as described for iodinations in the Materials and methods section (free, ——). A tracer amount of $^{131}$I-insulin was first added to the sample to mark the 6000 Da peak, and radioactivity for $^{125}$I-labelled material was corrected for the crossover of radioactivity for $^{131}$I-labelled material. The pellet from the centrifugation was resuspended in 500 μl of 0.01 M-HCl (+1 g of bovine serum albumin/litre) for 60 min to dissociate radioactivity from LPM. Sample was centrifuged and supernatant removed and chromatographed as above (bound, ····). (*d–f*), Controls consisted of labelled ligand chromatographed as above after no incubation (····) or incubation for 30 min at 37°C in the absence of LPM (——). Peaks refer to (I) void volume, (IIa) 12000 and (IIb) 6000 Da material and (III) low-molecular-weight products. Results shown are representative of three or four experiments with each ligand.

basis, implying that each monomeric half was theoretically as potent as an insulin monomer (i.e. equipotent with an equal mass of insulin). However, compared with an insulin monomer, each half of B1-B'29 D only retained about half of its ability to stimulate lipogensis (e.g., 40% potent on an equal mass basis), and for B1-B'1 D this was reduced to 15%. When DOP (cross-linked at B1) was substituted for the B1-cross-linked insulin half of B1-B'29 D (i.e., B29-B'1 DOP), binding decreased from 202 to 13% (a reduction of 93.39%) and lipogenesis from 79 to 8% (a reduction of 90.20%). This same substitution with either half of B1-B'1 D (i.e. B1-B'1 DOP) resulted in a decrease in binding from 199 to 93% (a reduction of 53.06%) and in lipogenesis from 29 to 21% (a reduction of 27.62%). These results suggested that the B1-cross-linked insulin half of an asymmetrically-linked dimer predominantly interacted with the receptor. This confirmed the proposed orientation of the monomeric halves in these dimers, i.e., a partial obstruction of the putative binding region of the B29-cross-linked half by the other half. Schematic diagrams of insulin and the five dimers are shown in Fig. 1, with the monomeric halves of each dimer rotated such that the receptor 'binding' regions are in the same plane.

*Degradation of labelled ligands*

Fig. 2 shows examples of gel-filtration elution profiles of labelled material unbound ('free') and dissociated from LPM ('bound') (*a–c*), and of unincubated and 37°C-incubated blanks (*d–f*). Incubation in HCl for 60min at room temperature produced no change in the elution profile of the tracer material (results not shown). With blanks incubated in buffer at 37°C the proportion of the total elution profile that eluted as labelled high-molecular-weight material (peak I) was greater with dimers than with insulin: 5.06 ± 0.53% for insulin, 16.00 ± 1.38% for B1-B'29 D and 14.68 ± 0.52% for B1-B'1 D ($n = 3$ for each; $P < 0.001$ for both dimers). Since these high-molecular-weight products were formed in the absence of LPM, data for 'free' and 'bound' samples were first corrected for the appropriate control and then LPM-associated degradation of each labelled ligand was calculated, as summarized in Table 2.

There were no significant differences in degradation between 'free' samples of all ligands. Degradation of insulin was significantly lower in 'bound' samples compared with 'free' ($P < 0.02$), in agreement with the observations of others (Gliemann & Sonne, 1978; Olefsky *et al.*, 1979). This same comparison could not be made with dimers because of the reduced efficiency of dissociation of dimers by HCl. Degradation was reduced by about 3-fold in the presence of 1 μM unlabelled insulin (e.g. non-specific binding conditions; results not shown). This has been reported by others for insulin (Gliemann & Sonne, 1978; Olefsky *et al.*, 1979). Under non-specific binding conditions 50% (insulin) and 71% (dimers) of bound labelled material was not dissociable by HCl (results not shown). Of the material extracted by HCl, 74% and 65% respectively appeared as intact ligand. The remainder was approximately equal amounts of labelled high- and low-molecular-weight products.

Table 2. *Membrane-associated degradation of labelled insulin and dimers*

The proportions of total radioactivity that eluted as either peak IIa (dimer) or IIb (insulin) in the experiments described in the legend to Fig. 2 were determined for free and bound samples and controls for each labelled ligand. These values were referred to as 'intact ligand'. Percentage membrane-associated degradation of each ligand was calculated by correcting the values for 'intact ligand' of free with those of the 37°C-incubated controls (i.e. a comparison of the solid lines in *a–c* with those in *d–f* of Fig. 2), and the 'intact ligand' of bound with those of the unincubated controls (the broken lines in *a–c* and *d–f* of Fig. 2), as follows:

$$\frac{\text{Intact ligand (control)} - \text{intact ligand (sample)}}{\text{intact ligand (control)}} \times 100\%$$

Values are means ± S.E.M. of three to four experiments with each labelled ligand.

| Ligand | Membrane-associated degradation (%) | |
|---|---|---|
| | Free | Bound |
| Insulin | 16.55 ± 2.09 | 5.57 ± 2.62 |
| B1-B'29 D | 24.16 ± 3.62 | 13.26 ± 5.88 |
| B1-B'1 D | 12.34 ± 2.00 | 3.38 ± 2.49 |

The degree of intactness of labelled material in peak IIa or IIb was assessed by rebinding to fresh LPM (Table 3) and by total immunoprecipitation with anti-insulin serum (results not shown). The specific binding fractions of material in peaks IIa or IIb (of all three ligands) for LPM and for insulin antibodies were the same as those of the original tracer material. Table 3 shows that for each ligand, the specific binding to LPM of material from peak I was markedly lower than that of peak II material ($P < 0.002$ for insulin and B1-B'29 D, $P < 0.01$ for B1-B'1 D), whereas the non-specific binding of material from both peaks remained similar. The percentage of total bound that was non-specific was greater for dimers than for insulin [11.3 ± 3.1% for insulin, 27.9 ± 7.1% for B1-B'29 D ($P < 0.001$) and 21.5 ± 5.7% for B1-B'1 D ($P < 0.05$). Binding of material in peak III (results not shown) was in all cases less than 10% of that of intact tracer, and 98% of this binding was non-specific.

Table 3. *Binding properties in LPM of degradation products of labelled insulin and dimers*

Similar amounts of radioactivity of freshly thawed labelled ligand (control) and samples from peaks I, IIa or IIb from the degradation experiments described in the legend to Fig. 2 were incubated with LPM (200 $\mu$g/ml) for 60–75 min at 30°C in the absence or in the presence of 1 $\mu$mol of unlabelled insulin/litre to determine specific and non-specific binding. Data are means ± S.E.M. for the numbers of experiments shown in parentheses.

| | Proportion bound (%) | | |
|---|---|---|---|
| | Insulin | B1-B'29 D | B1-B'1 D |
| Control | (7) | (4) | (3) |
| Specific | 7.41 ± 0.99 | 9.17 ± 1.50 | 5.27 ± 1.32 |
| Non-specific | 0.89 ± 0.14 | 4.35 ± 0.22 | 1.10 ± 0.31 |
| Peak II (a or b) | (7) | (5) | (4) |
| Specific | 7.73 ± 1.08 | 8.78 ± 1.24 | 6.64 ± 0.70 |
| Non-specific | 1.16 ± 0.23 | 4.64 ± 0.49 | 1.22 ± 0.30 |
| Peak I | (5) | (5) | (4) |
| Specific | 2.42 ± 0.59 | 1.10 ± 0.49 | 0.62 ± 0.23 |
| Non-specific | 2.43 ± 0.78 | 5.14 ± 1.25 | 2.64 ± 0.68 |

Table 4. *Observed and predicted binding potencies of dimers*

LPM binding data observed with the DOP-dimers were used to derive 'expected' values for the I-I dimers. In this calculation the non-DOP half in DOP-dimers was assumed to contribute 100% of the activity of the dimer. Values so derived for monomeric halves in DOP-dimers were then attributed to the analogous monomeric halves in the I-I dimers and the 'expected' potency of each I-I dimer was calculated by addition of the two contributing monomeric potencies. The theoretical basis of the discrepancies between observed and expected values is discussed in the text. Ratios of observed to expected values are in the right-hand column.

| | Binding potencies (%) | | Observed |
|---|---|---|---|
| Dimer | Observed | Expected | Expected |
| B1-B'1 D | 200 | 93 + 93 = 186 | 1.1 |
| B1-B'1 DOP | 93 | — | |
| B1-B'29 D | 200 | 93 + 13 = 106 | 1.9 |
| B29-B'1 DOP | 13 | — | |
| B29-B'29 D | 74 | 13 + 13 = 26 | 2.8 |

## Discussion

DOP insulin has been reported to have intrinsic binding and biological potencies of 0.1% (Kikuchi *et al.*, 1980). This low activity is probably due to its almost complete lack of the putative binding region. Although the markedly reduced affinity of DOP insulin has not been explained in terms of its binding kinetics, it is a fair assumption that with a 0.1% affinity the DOP half of a dimer does not readily bind to the receptor. It follows that the activity of a DOP-dimer will be a reflection of the intact insulin half of that dimer. If, in addition, we can show that I-I dimers bind only univalently, the observed potencies of the DOP-dimers should allow estimation of the contribution made by each half of an I-I dimer. These values for observed (from LPM assays shown in Table 1) and predicted binding potencies are shown in Table 4.

With a bivalent ligand that is capable of occupying two binding sites simultaneously, the binding of the first half to a receptor would create a concentrating effect on the unbound half in the vicinity of the cell surface and hence of the receptor sites. This phenomenon has been termed the localization factor of the ligand (Minton, 1981), and the affinity of this ligand would be many orders of magnitude greater than the sum of the affinities of the halves, i.e. the product of the two affinities. We can regard the ratios of the observed to 'expected' binding potencies of the I-I dimers in Table 4 as an index of the magnitude of their localization factors. The small ratios (1.1–2.8) suggest that dimers have a potential for bivalency, but rule out true bivalent binding of dimers. Further support for univalent binding of dimers is discussed in a previous publication on the effect of alteration in pH on binding of labelled insulin and dimers (Tatnell & Jones, 1981).

Piron *et al.* (1980) had proposed a tetravalent receptor model from their initial binding data for six covalent I-I dimers with cultured human IM-9 lymphocytes. However, their model required a re-organization of the quaternary structure of the receptor to accommodate the proposed orientations of all the dimers during binding. Numerous studies on the molecular structure of the receptor complex have provided powerful evidence that the receptor consists of no more than two binding components (Massague *et al.*, 1980; Jacobs *et al.*, 1980; Yip *et al.*, 1982). Let us consider how the localization factors of the I-I dimers (Table 4) and the orientation of the putative receptor binding regions within each dimer (Fig. 1) might provide an insight into the subunit structure and valency of the receptor complex.

With univalent binding of dimers, a localization factor increases the probability of binding of the unbound half to the same or to a nearby site, after the other half dissociates. If receptors were univalent, B1-B'29 should have the highest localization factor, because of the parallel symmetry of its monomeric halves. However, this was not the case, as the localization factors for B29-B'29 D, B1-B'29 D, and B1-B'1 D were 2.8, 1.9, and 1.1 respectively. It is interesting to note that the distance between the two putative binding regions within an I-I dimer and the degree of flexibility between monomeric halves of each dimer are inversely proportional to the magnitude of the localization factors of these dimers. The *N*-terminus of the B chain of insulin (B1–B7) is known to be very flexible and capable of extending

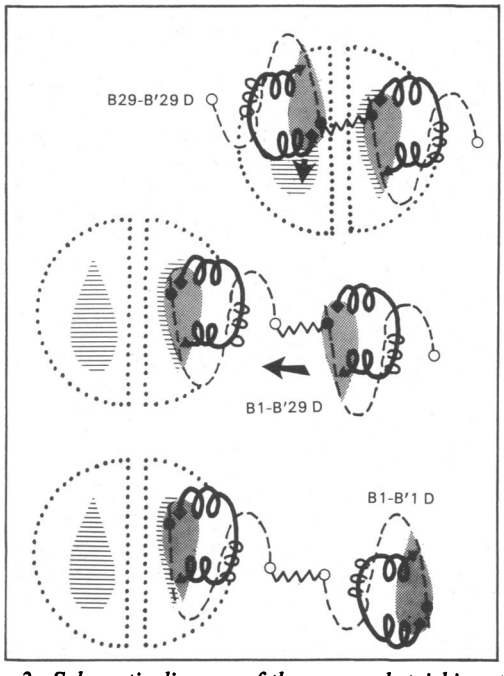


Fig. 3. *Schematic diagram of the proposed stoichiometry of two binding sites within the insulin receptor and the orientation of the three insulin–insulin dimers during the binding event*

The dimers are rotated such that their 'binding' regions (shaded areas) are in the same plane, as in Fig. 1. The anti-parallel symmetry of the residues of two binding subunits that are involved in hormone–receptor interaction are represented by the hatched areas. The arrow indicates the probable direction of binding of the unbound half of a dimer, once the first half dissociates.

away from the main body of the molecule, whereas the *C*-terminus of the B chain is constrained, particularly by the salt bridge between the carboxy side chain of $Ala^{B30}$ and the amino group of $Gly^{A1}$ (Dodson *et al.*, 1979).

These considerations suggest that the 2-fold symmetry implicit in the more rigid B29–B'29 linkage matches the structure of the receptor more closely than that in either of the other two dimers, i.e. the receptor complex consists of two binding subunits, with an anti-parallel symmetry of the residues of the sites involved in interaction with insulin. Further studies to attempt to delineate the distance between binding sites are in progress using B29-B'29 D derivatives in which the covalent linkage has been varied in length. The proposed orientations of the three I-I dimers during univalent binding to bivalent receptor complexes and the stoichiometry of the receptor binding subunits are schematically illustrated in Fig. 3. Note that once the first half of a dimer dissociates, the ease with which the second half can bind (i.e. the localization factor) is greatest with B29-B'29 D and least with B1-B'1 D.

Lower biological responses than expected from binding potencies cannot be ascribed to bivalent binding of dimers. Moreover, these observations do not appear to correlate directly with the binding kinetics of dimers. Compared with labelled insulin the labelled I-I dimers possessed slower binding kinetics for both LPM and adipocytes (Willey *et al.*, 1980; M. A. Tatnell, unpublished work). However, the kinetics of B1-B'1 D were the slowest and those of B29-B'29 D the most similar to those of insulin, yet both dimers had larger discrepancies between binding and biological effects (5.6- and 6.6-fold respectively) than did B1-B'29 D (2.5-fold). Such discrepancies also cannot be ascribed to differences in rates of degradation, as the degradation of dimers was found not be be markedly different from that of insulin. Labelled dimers showed a 3-fold greater proportion of high-molecular-weight material at 37°C in the absence of LPM, compared with labelled insulin. Since these aggregates bind with low affinity, this may account in part for the higher non-specific binding of $^{125}$I-dimers compared with that of $^{125}$I-insulin. They may also not induce a biological response, although allowance for this still does not explain the discrepancies observed between binding and biological potencies.

With a bivalent receptor it is possible that the true binding affinities of dimers are only half their measured binding potencies if the unbound half of a univalently-bound dimer obstructs the adjacent unoccupied receptor binding site. For example, in competition binding experiments, one dimer with approximately the same affinity as insulin inhibits the binding of two $^{125}$I-insulin molecules by occupying one binding site but obstructing an adjacent site by steric hindrance. On the other hand, one insulin molecule occupies one binding site, inhibits one $^{125}$I-insulin molecule and transmits one biological signal. Thereby a dimer would be equipotent with an equimolar amount of insulin in inducing a biological response, but apparently twice as potent in binding to receptors. This might account for the 2-fold difference between binding and lipogenic potencies of B29-B'1 DOP, but cannot totally explain the 2.5–6.6-fold discrepancies of the other four dimers (Table 1).

A more plausible role of steric hindrance to account for the discrepant biological properties of dimers is interference by the bulky unbound half of a dimer in events during binding that are essential for full biological expression. These events are proposed to include a conformational change either within the receptor complex and/or in the insulin molecule. The occurrence of such conformational changes has recently received considerable support by others

from crystallographic studies on the various tertiary conformations assumed by the insulin monomer in different crystalline forms (Cutfield *et al.*, 1979; Dodson *et al.*, 1983), from studies on the structural and functional organization of the insulin receptor (Pilch & Czech, 1980; Berhanu *et al.*, 1983; Van Obberghen *et al.*, 1983) and from a thermodynamic analysis of the insulin–receptor interaction (Waelbroeck *et al.*, 1979). Fig. 3 shows that with B1-B'29 D and B1-B'1 D there appears to be only a small difference in the position of the unbound half relative to the two binding subunits of the receptor despite their markedly different discrepancies in binding and biological potencies (2.5- and 6.6-fold respectively). This suggests that the exact orientation of the unbound half of a dimer might be critical in determining the degree of steric hindrance in the proposed required conformational change. As apparent partial competitive antagonists of insulin, covalent insulin dimers would prove useful in identifying the relationship between the coupling of insulin to its receptor and the initial biochemical event in insulin action.

We thank Dr. J. Pitts (Birkbeck College, London, U.K.) and Dr. G. G. Dodson (University of York, York, U.K.) for their individual stimulating discussions on some of these results. This work was financially supported by The Wellcome Trust, London, St. Thomas' Hospital Research (Endowments) Committee and The Juvenile Diabetes Federation.

## References

Berhanu, P., Tsai, P. & Saunders, D. (1983) *Diabetes* **32**, 2A

Blundell, T. L., Dodson, G. G., Hodgkin, D. & Mercola, D. (1972) *Adv. Protein Chem.* **26**, 279–402

Burke, G. T., Chanley, J. D., Okada, Y., Cosmatos, A., Frederigos, N. & Katsoyannis, P. G. (1980) *Biochemistry* **19**, 4547–4556

Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 7265–7274

Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Emdin, S. F. & Reynolds, C. D. (1979) *J. Mol. Biol.* **132**, 85–100

Cutfield, J., Cutfield, S., Dodson, E. J., Dodson, G. G., Hodgkin, D. C. & Reynolds, C. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 755–761

Dodson, E. J., Dodson, G. G., Hodgkin, D. C. & Reynolds, C. D. (1979) *Can. J. Biochem.* **57**, 469–479

Dodson, E. J., Dodson, G. G., Hubbard, R. E. & Reynolds, C. D. (1983) *Biopolymers* **22**, 281–291

Finney, D. J. (1964) in *Statistical Methods in Biological Assays*, 2nd edn. Griffin and Co., London

Freychet, P., Brandenburg, D. & Wollmer, A. (1974) *Diabetologia* **10**, 1–5

Gliemann, J. (1967) *Diabetologia* **3**, 382–388

Gliemann, J. & Gammeltoft, S. (1974) *Diabetologia* **10**, 105–113

Gliemann, J. & Sonne, O. (1978) *J. Biol. Chem.* **253**, 7857–7863

Gliemann, J., Osterlind, K., Vinten, J. & Gammeltoft, S. (1972) *Biochim. Biophys. Acta* **286**, 1–9

Greenwood, F. C., Hunter, W. H. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123

Horuk, R., Blundell, T. L., Lazarus, N. R., Neville, R. W. J., Stone, D. & Wollmer, A. (1980) *Nature (London)* **286**, 822–824

Jacobs, S., Hazum, E. & Cuatracasas, P. (1980) *J. Biol. Chem.* **255**, 6937–6940

Kikuchi, K., Larner, J., Freer, R. J., Day, A. R., Morris, H. & Dell, A. (1980) *J. Biol. Chem.* **255**, 9281–9288

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Massague, J., Pilch, P. F. & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7137–7141

Minton, A. P. (1981) *Mol. Pharmacol.* **19**, 1–14

Moody, A. J., Stan, M. A., Stan, M. & Gliemann, J. (1974) *Horm. Metab. Res.* **6**, 12–16

Olefsky, J. M., Kobayashi, M. & Chang, H. (1979) *Diabetes* **28**, 460–471

Peking Insulin Structure Research Group (1974) *Scientia Sinica* **17**, 752–778

Pilch, P. F. & Czech, M. (1980) *Science* **210**, 1152–1153

Piron, M. A., Michiels-Place, M., Waelbroeck, M., DeMeyts, P., Schüttler, A. & Brandenburg, D. (1980) in *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D. & Wollmer, A., eds.), pp. 371–391, DeGruyter, Berlin and New York

Posner, B. I., Josefsberg, Z. & Bergeron, J. J. M. (1978) *J. Biol. Chem.* **253**, 4067–4073

Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J. & Gammeltoft, S. (1976) *Nature (London)* **259**, 369–373

Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380

Rösen, P., Simon, M., Reinauer, H., Friesen, H. J., Diaconescu, C. & Brandenburg, D. (1980) *Biochem. J.* **186**, 945–952

Sasaki, K., Dockerill, S., Adamiak, D. A., Tickle, I. J. & Blundell, T. L. (1975) *Nature (London)* **257**, 751–757

Schlütter, K., Petersen, K. G., Schüttler, A., Brandenburg, D. & Kerp, L. (1980) in *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D. & Wollmer, A., eds.), pp. 433–438, DeGruyter, Berlin and New York

Schüttler, A. & Brandenburg, D. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 317–330

Tatnell, M. A. & Jones, R. H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1315–1321

Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 945–949

Waelbroeck, M., Van Obberghen, E. & DeMeyts, P. (1979) *J. Biol. Chem.* **254**, 7736–7740

Willey, K. P., Tatnell, M. A., Khalili-Shirazi, A., Jones, R. H., Thomas, S. H. L., Schüttler, A. & Brandenburg, D. (1978) *Diabetologia* **14**, 281

Willey, K. P., Tatnell, M. A., Jones, R. H., Schüttler, A. & Brandenburg, D. (1980) in *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D. & Wollmer, A., eds.), pp. 425–431, DeGruyter, Berlin and New York

Wisher, M. H. & Evans, W. H. (1975) *Biochem. J.* **146**, 375–388

Wisher, M. H., Dron, D. I., Sönksen, P. H. & Thomas, J. H. (1977) *Biochem. Soc. Trans.* **5**, 313–316

Yip, C. C., Moule, M. L. & Yeung, C. W. T. (1982) *Biochemistry* **21**, 2940–2945