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An examination of the role of insulin dimerisation and negative cooperativity using the biological properties of the despentapeptide and deshexapeptide insulins

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Summary. The C-terminus of the insulin B chain is essential for dimerisation and expression of negative cooperativity. In order to evaluate the possible physiological role of these phenomena, we have studied the properties in vivo and in vitro of despentapeptide insulin (B26-30 deleted), derived from beef insulin, and deshexapeptide insulin (B25-30 deleted), derived from pork insulin. These materials do not dimerise and have 15% and 0% retention of negative cooperativity respectively. Lipogenesis potencies in rat adipocytes were: despentapeptide insulin 19.9±0.3%; deshexapeptide insulin 19.9±1.5%. Binding potencies in adipocytes were: despentapeptide insulin 22.6±7.8%; deshexapeptide insulin 13.2±3.3%. Metabolic clearance rates were reduced compared to insulin (insulin = 19.1±0.9; despentapeptide insulin = 9.7±0.8; deshexapeptide insulin = 6.4±0.6 $ml \cdot min^{-1} \cdot kg^{-1}$ at plasma concentration 0.5 nmol/l). Hypoglycaemic potencies were reduced for both analogues (40% and 30%) when calculated on the basis of plasma concentration although both analogues and insulin were equally effective at lowering plasma glucose concentration in equimolar doses. Plasma half-disappearance time was prolonged (despentapeptide insulin = 7.3±0.5; deshexapeptide insulin = 9.1±0.2 min). Both analogues were full agonists and conformed to the general relationship between in vitro and in vivo properties seen with a wide range of modified insulins. They resemble other analogues with modifications which reduce receptor affinity without impairing dimerisation or negative cooperativity. The results do not support a physiological role for dimerisation or negative cooperativity.

Key words. Insulin, despentapeptide insulin, deshexapeptide insulin, negative cooperativity, insulin demerisation, lipogenesis, insulin binding, insulin metabolism.

The binding of insulin to its receptor is a complex phenomenon. It does not follow the simple law of mass action since Scatchard analysis of binding data gives curvilinear plots, suggesting either several classes of receptor with differing affinities, or an ability to change affinity with varying insulin receptor occupancy. This latter explanation has been evolved into the negative cooperativity concept and evoked most forcefully by de Meyts et al [1]. According to this concept the dissociation rate of insulin from its receptor increases with increasing receptor occupancy. This leads to the conclusion that the insulin receptor can exist in different affinity states according to the insulin concentration to which it is exposed. Evidence for the existence of this phenomenon in vitro is strong, particularly in IM9 lymphocytes, but its relevance to the action of insulin in the intact organism remains in doubt. Studies with different species of insulin and with chemically-modified insulins have suggested that a discrete region on the surface of the insulin monomer is essential for the expression of negative cooperativity. This region comprises the 8 C-terminal residues of the B chain together with A21 asparagine. Thus desoctapeptide insulin (DOP) and desalanine desasparagine insulin (DAA) are non-cooperative and analogues with deletions of 4 or more residues from the C terminus of the B chain show markedly impaired ability to induce negative cooperativity [2]. This region of the insulin molecule also forms part of the dimer-forming surface. It has been argued that the burial of the region by dimerisation also leads to a loss of negative cooperativity. In addition it has been suggested that dimerisation per se at the receptor may be an important initial step in the mechanism of insulin action [3].

In an attempt to evaluate the importance of negative cooperativity and dimerisation to the action of insulin in vivo we have chosen to study the biological properties of two chemically modified insulins, despentapeptide insulin (DPI) and deshexapeptide insulin (DHI) with deletions of five and six amino acids, respectively, from the C terminus of the B chain. These analogues retain significant binding affinity for the receptor and biological potency but, as predicted from the crystal structure, DPI (and by implication DHI) are

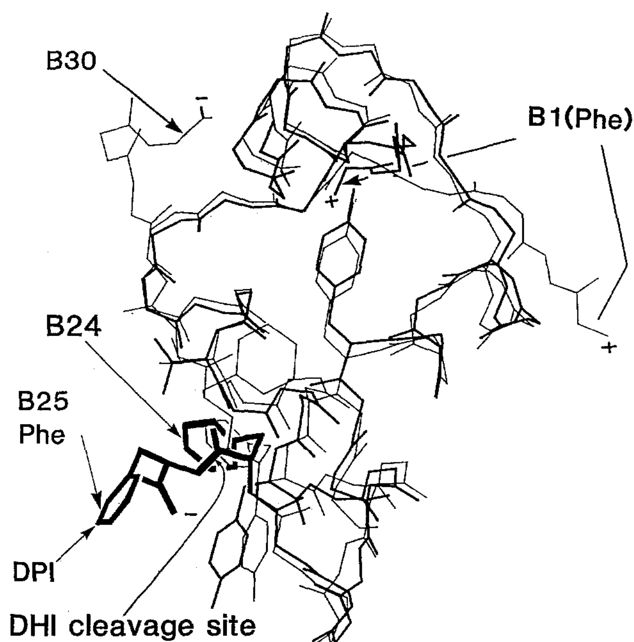

Fig. 1. A 2-dimensional representation of the crystallographic structure of 2 Zn porcine insulin (thin lines) and beef despentapeptide insulin (DPI) (thick lines). The positions of the B 24 and B 25 residues are shown by the exaggerated thick lines

unable to form dimers [3, 4]; and both analogues display a markedly impaired capacity to induce negative cooperativity [2, 5].

The crystal structure of DPI (bovine, porcine and ovine) has been determined. The structures of DPI and insulin are similar (6-8) and are illustrated in Figure 1. The major differences occur at B1-B3 and at B25 which is the B chain terminal amino acid in DPI. In DPI the B25 phenylalanine residue is directed into solution and makes no hydrogen bond or other contact back to the molecule. This suggests that the removal of B25 phenylalanine will not affect significantly the structure of the molecule and therefore DHI and DPI will have very similar structures. This point is demonstrated in Figure 1. Furthermore the comparable potency of these two monomeric insulins in vitro indicates that B25 phenylalanine is not critically involved in receptor binding and the expression of activity.

Materials and methods

Repurified bovine insulin was kindly supplied in lyophilised form by Professor D. Brandenburg (Deutsches Wollforschungsinstitut, Aachen, FRG). After reconstitution at acid pH the exact concentration was checked before use by absorption spectrophotometry at 276 nm. Despentapeptide insulin (bovine) was prepared by controlled pepsin cleavage at the B25/B26 bond using pepsin at 4 °C according to the method of Gattner [9] and was purified by column chromatography. DPI was crystallised and the X-ray diffraction pattern shown to be that of DPI [8]. Crystalline deshexapeptide insulin (porcine) was prepared by trypsin cleavage of insulin to yield desoctapeptide insulin followed by enzymatic semisynthesis [10]. Seperation from insulin and desoctapeptide insulin was by DEAE-Sephadex A25 column chromatography. Purity was confirmed by analysis of amino acid composition [10], by polyacrylamide gel electrophoresis, by high performance liquid chromatography [11] and by comparison of CD spectra. The concentration of DPI and DHI (received in lyophilised form and reconstituted) was checked by absorption spectrophotometry at 276 nm, a correction being made for the absence of the B 26 tyrosine.

For each analogue lipogenesis and binding potencies compared with bovine insulin were estimated in isolated rat adipocytes. Hypoglycaemic potency, metabolic clearance rate (MCR), apparent distribution space (DS) and plasma half disappearance time (t1/2) were derived from experiments in greyhound dogs.

Lipogenesis

Lipogenesis was measured as previously described [12].

Isolated fat cells were prepared by a modification [13] of the method of Rodbell [14]. Aliquots of the cell pool in buffer were incubated with glucose (0.55 mmol/l) and 3-^{3H}-glucose (0.1 µCi, Radiochemical Centre, Amersham, UK). Serial dilutions of bovine insulin, DPI and/or DHI were included in each assay and incorporation of glucose-derived ^{3}H into toluene-extractable lipid was determined [15].

Potency estimates for each analogue compared with insulin were obtained from the linear portion of the log-dose response curves using the parallel-line bioassay technique described by Finney [16]. This method incorporates a check for parallelism.

Fat cell binding

Isolated fat cells were prepared as described above. Serial dilutions of bovine insulin, DPI or DHI were added to the incubation vials containing aliquots of the cell suspension. The radioligand used was ^{125}I insulin prepared by the chloramine T technique using a 10:1 molar ratio of chloramine T to insulin and purified by discontinuous polyacrylamide gel electrophoresis using a modification of the method described by Linde et al. [17]. The material used was obtained from the peak corresponding to A14-monoiodoinsulin [17]. The incubation period was 60 min at 30 °C. Bound and free radioligand were then separated by centrifugation after the addition of dinonylphthalate to triplicate 200 µl samples of incubation mixture placed in microfuge tubes. The resulting competition binding curves were again analysed following the method of Finney [16], so that potency estimates could be obtained.

Activity and metabolism in vivo

Experiments were performed in intact, anaesthetised greyhound dogs using the priming-dose, stepped infusion technique following published methods [18]. Bovine insulin, DPI and DHI were infused to achieve four separate steady state plasma concentrations spanning the physiological range for insulin. During each 30-min steady state period blood samples were collected at 5, 10, 20 and 30 min for measurement of serum insulin or analogue and plasma glucose concentrations. After cessation of the infusion blood was withdrawn at 1-min intervals for 10 min, 5-min intervals for 20 min and then again at 10-min intervals until 1 h had elapsed. This allowed accurate delineation of the resulting decay curve.

Insulin and analogue concentrations were determined by double-antibody radioimmunoassay [19]. Although the antiserum used (raised in guinea-pigs) gave indistinguishable standard curves with insulin and both analogues, analogue samples were assayed against standard curves obtained using the same analogue rather than the native hormone.

Plasma glucose was measured by the glucose oxidase technique using a Clandon Scientific model 23 AM glucose analyser (Yellow Springs Instrument Co., Clandon Scientific, London, UK).

MCR and DS were calculated as described previously [20]. For MCR estimation the steady state concentration used in the calculation was the mean of the four measured values from each period of the experiment. T1/2 values were obtained graphically. Hypoglycaemic potency was calculated according to a method that allows comparison of hypoglycaemic effect with steady-state plasma insulin or

analogue concentration rather than with dose administered, the main aim being to correct for slowed metabolism. A full description of this method has been previously published [18]. In essence the method compares the fall in blood glucose from baseline at the end of a steady-state period with the prevailing steady-state concentration of insulin/analogue. A log dose-response relationship is then constructed and subjected to parallel line bioassay analysis, again following the method of Finney [16], thereby allowing estimation of mean potency with 95% fiducial limits.

Statistical analysis

Statistical comparison between insulin and analogue binding and lipogenesis potencies was by paired t-test whereas an unpaired test was used for comparison of MCR, DS and t1/2.

Results

Bioactivity in vitro

Examples of the relative abilities of DHI and bovine insulin to stimulate lipogenesis and bind to adipocytes are shown in Figures 2 and 3. In these examples the estimated potency of DHI relative to bovine insulin were 16% for binding and 20% for lipogenesis. Pooled data from all experiments with each analogue are summarised in Table 1.

Metabolism

MCR values at each steady state concentration are summarised in Figure 4 and demonstrate a marked reduction for both analogues compared with native insulin. To enable statistical comparison of these results the MCR values at a plasma concentration of 0.5 nmol/l have been obtained graphically for each individual experiment and meaned. The results (Table 1) show a statistically significant ($p < 0.001$) reduction in the rate of metabolism for both analogues, with the impairment of metabolism being more marked in the case of DHI. It can also be seen from Table 1 that t1/2 values are significantly lengthened (DHI, $p < 0.001$; DPI, $p < 0.01$). However DS values for both analogues do not differ significantly from those of insulin.

Bioactivity in vivo

Figure 5 shows that the plasma glucose response to infusion of equimolar quantities of all three materials is indistinguishable. However when the hypoglycaemic effect is related to plasma concentration (see Methods) the potency of both analogues with respect to bovine insulin can be seen to be reduced (Table 1).

Relationship between in vitro potency and metabolism

We have previously reported the existence of a close relationship between the vitro binding and lipogenesis potencies, and MCR for a range of chemically modified monomeric insulin derivatives [12, 20]. Figure 6 summarises these findings (for lipogenesis only) and confirms that both DPI and DHI conform to this general relationship.

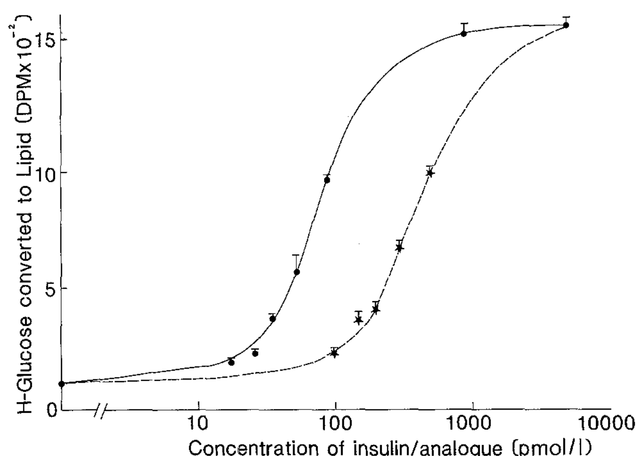

Fig. 2. Lipogenesis in isolated rat adipocytes. Log dose-response curves comparing deshexapeptide insulin (DHI) (★-----★) to bovine insulin (●—●). The dose points are the mean of triplicates and the vertical bar represents the SE. According to Finney analysis of the parallel section of the dose-response curves the potency estimate for DHI relative to insulin is 20%

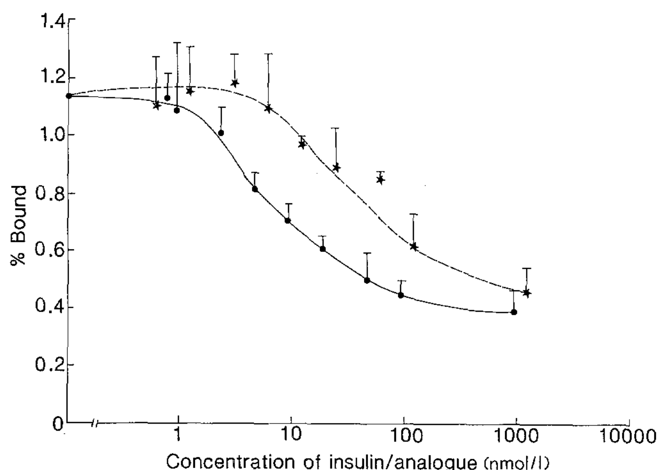

Fig. 3. Binding to isolated rat adipocytes. Competition binding curves comparing DHI (★-----★) to bovine insulin (●—●). Data points are mean + SE of triplicates. Finney analysis yields a potency estimate of 15.5% for DHI relative to bovine insulin

Discussion

The results confirm that the insulin analogues DPI and DHI have reduced biological activity both in vitro and in vivo. The in vitro potency of 19.9% for DPI is in agreement with that reported by most other groups (15-35%) [21]. Interestingly, however, despentapeptide insulinamide, in which the charged B25 C-terminal carboxylate is neutralised, has been reported to be equipotent with native insulin in vitro [21]. This emphasises the relevance of the DPI structure (Fig. 1) to understanding the active surfaces of the monomer and their relationship to the intact hormone.

The in vitro potency of DHI is higher than the reported values of Cui et al. [22] and Rieman et al. [23]. The reason for this discrepancy is unclear. Rieman et al. [23] report a binding potency of 6.3% and lipogenesis potency of 8% using DHI derived from bovine in-

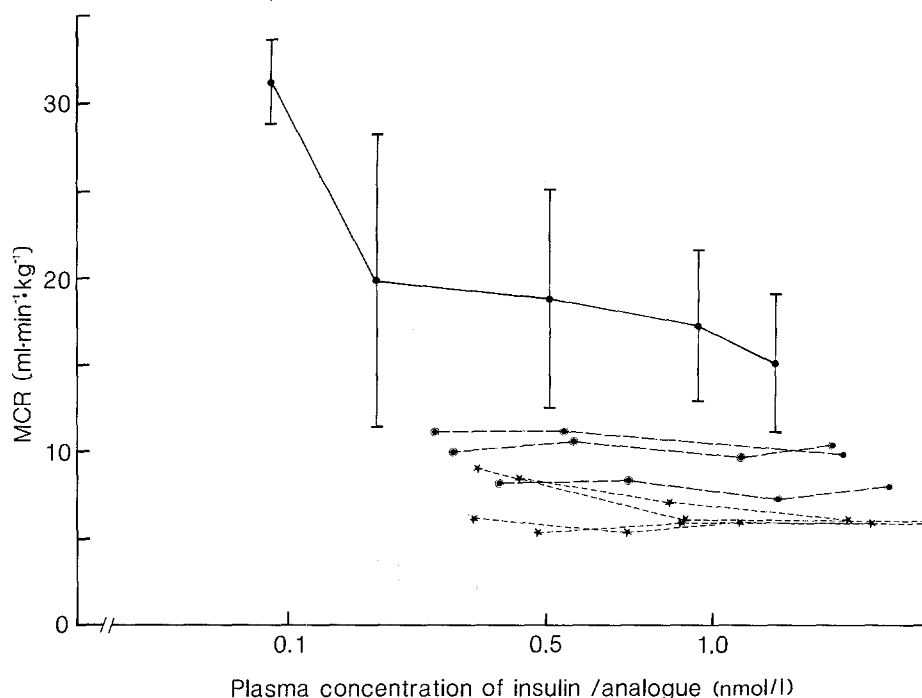

Fig. 4. Metabolic clearance rate (MCR) ($ml \cdot min^{-1} \cdot kg^{-1}$) at a range of steady state plasma concentrations (nmol/l). The solid line represents the mean of 9 experiments with bovine insulin. The vertical bars represent the range of normal values as expressed by 2 SD on either side of this mean. 3 individual experiments with DPI (●-----●) and 4 experiments with DHI (★-----★) are shown for comparison

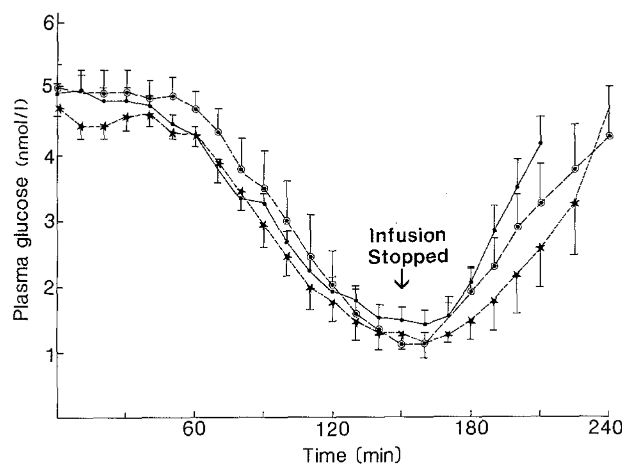

Fig. 5. Plasma glucose response to the stepped infusion of bovine insulin (●—●, mean ± SE, n=9), DPI (⊙-----⊙, mean ± SE, n=3) and DHI (★-----★, mean ± SE, n=4). The arrow indicates the time at which the insulin was discontinued

sulin. However, the species difference seems unlikely to explain the lower potency since Cui et al. using porcine DHI report a still lower potency of 2% for binding to human placental membrane. Contamination with desoctapeptide insulin is a theoretically possible explanation for this lower potency but is highly unlikely to explain a difference of this magnitude, particularly since the analogues have been carefully characterised before use. Differences in assay methodology are also a possible mechanism for different potencies and neither report states the number or experiments performed, the number of replicates used or gives an indication of the variance of the data.

It is important to note that there is no discrepancy between binding potency to adipocyte receptors and biological effect in adipocytes as assessed by lipogenesis. Both analogues are also full agonists. In addition the reduction of MCR is commensurate with the reduction of lipogenesis potency and conforms to the general relationship noted with a wide range of chemically-modified insulins which includes insulin analogues with single amino acid alterations in the A chain [12] and analogues with modifications at the N terminus of the B chain, the N terminus of the A chain and the C terminus of the B chain [18, 24–26]. At none of these sites are the modifications thought to interfere either with demerisation or with the ability to induce negative cooperative effects.

The greater hypoglycaemic potencies than in vitro potencies noted with both DPI and DHI, despite correction for plasma concentration, could result from secretion of counterregulatory hormones which may partially reduce the hypoglycaemic effect of native insulin compared with the lower potency analogues. A similar discrepancy has been noted in our laboratory with some, but not all, insulin analogues of comparable potency, for example proinsulin, Al-thiazolidine insulin and A1-B29 dodecoyl insulin [25].

The indistinguishable hypoglycaemic effect of DPI, DHI and native insulin, when no correction is made for slowed metabolism, explains the finding that DPI has the full biological activity of the native hormone when tested by the mouse convulsion assay [27], and agrees with our own observations using a wide range of insulin analogues [12, 18, 20]. The in vivo potency of porcine DHI reported by Cui et al. [22] is 40% using the mouse convulsion test which is not slightly higher than our value based on plasma concentration. In view of the comparative findings with DPI this value is perhaps lower than expected. However, as discussed above they also report a lower binding potency of 2%.

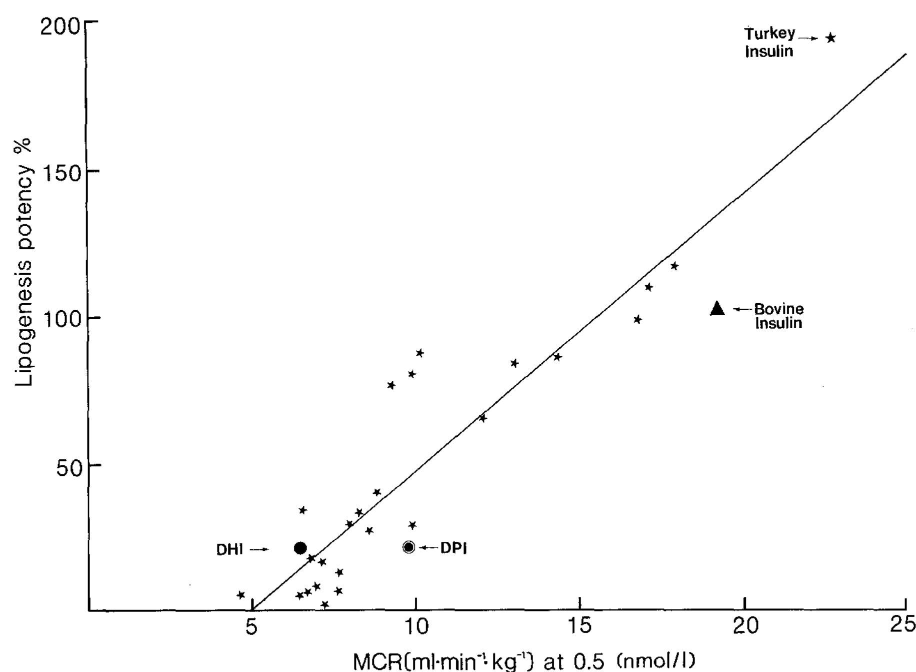

Fig. 6. The relationship between lipogenesis potency (%) and MCR at a plasma concentration of 0.5 nmol/l for a range of chemically-modified insulins. (▲ = bovine insulin, ⊙ = DPI, ● = DHI). Linear regression of these data gives $r = 0.94$, $p < 0.001$. Turkey insulin (★), with an in vitro potency of 200% is indicated for comparative purposes

Table 1. Summary of the biological properties of insulin, despentapeptide insulin and deshexapeptide insulin

	Bovine insulin	Despentapeptide insulin	Deshexapeptide insulin
Lipogenesis potency (%)	100	19.9 ± 0.3^{c} (4)	19.9 ± 1.5^{c} (3)
Binding potency (%)	100	22.6 ± 7.8^{c} (4)	13.2 ± 3.3^{c} (3)
Hypoglycaemic potency (%)	100 (9)	40.7 (3)	31.3 (4)
Metabolic clearance rate at plasma concentration 0.5 nmol/l (ml·min^{-1}·kg^{-1})	19.1 ± 0.9 (9)	9.7 ± 0.8^{c} (3)	6.4 ± 0.6^{c} (4)
Plasma half-life (min)	4.5 ± 0.4 (9)	7.3 ± 0.5^{b} (3)	9.1 ± 0.2^{c} (4)
Apparent distribution space (ml·kg^{-1})	112.0 ± 13.0 (9)	97.4 ± 9.1 (3)	74.5 ± 1.8 (4)

Values given as mean ± SEM, with the exception of hypoglycaemic potencies. The number of experiments is shown in brackets. Ninety-five per cent fiducial limits for hypoglycaemic potencies obtained by Finney Analysis were 25–66% for despentapeptide insulin and 19.6–49.9% for deshexapeptide insulin. The superscripts a, b and c show statistically significant differences to insulin ($a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$). The metabolic clearance rate of deshexapeptide insulin is also significantly lower than that of despentapeptide insulin ($p < 0.005$) and the plasma half life significantly longer ($p < 0.05$)

Thus the loss of dimerising capacity has left DPI and DHI with the same pattern of biological behaviour as other insulins with reduced potency but in which dimer formation and ability to induce negative cooperativity are relatively preserved. These observations demonstrate that the residue B25 phenylalanine, essential for dimer formation and for generating negative cooperativity, can be removed without disturbing the inherent biological properties of the insulin molecule. The conformation of B25 phenylalanine within the DPI molecule, where it is free of contacts to the molecule, makes it understandable that it can be removed without further disturbing the three-dimensional structure of the molecule.

The conclusion can be reconciled with the reported very low potency of B25-leucine insulin [28, 29] if phenylalanine but not leucine has the correct stereo properties to allow a necessary conformational change either before or during binding. This may also explain the low potency (4%) observed when the D-isomer of phenylalanine is substituted at the B25 position [30]. By contrast when B24 phenylalanine, whose conformation is the same in native insulin and DPI, and presumably DHI, is substituted by the D-isomer potency in vitro increases to 180% [30]. This finding is consistent with the possibility of conformational changes at the B-chain C-terminus, like those seen at B25 in DHI, actually extending further to B24 phenylalanine before or during the binding event.

Thus although there seems no doubt that residues B24–B26 are important in the expression of the biological effects of insulin [4, 31–33] and are involved in both dimerisation and cooperative effects there is no evidence that these processes are interdependent. The suggestion that these residues must conform to a necessary structural change following interaction with the receptor may explain the observation that structural changes in solution produced by modifications at B24 and B25 do not correlate with the alterations in biological activity [34].

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