The role of the arginine-B22 residue in insulin action

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We describe the modification of the side chain of the arginine-B22 residue of insulin by the N^8N^9 -(1,2-dihydroxycyclohex-1,2-ylene) group and by the adipoyl group. These are the first insulin derivatives described that contain a modified arginine residue in an otherwise unaltered molecule. When tested for their ability to lower blood sugar concentration, both modified insulins showed a specific activity indistinguishable from that of insulin. In view of the fact that the substituent groups involved are very bulky and in one case of opposite charge to that of the side chain, the retention of biological activity casts doubt on the idea, previously generally accepted, that the arginine-B22 residue is essential to the activity of the hormone.

One approach to the development of a long-acting form of insulin is to attach to the hormone groups that, by virtue of their bulk, charge distribution or hydrophobic nature, interfere with the action of the hormone. This attachment should be reversible under physiological conditions and the liberated moieties should be biodegradable. As a first step in this direction, we studied the reversible reaction between insulin and a functional group that might be employed to attach the interfering agents via the guanido group of the arginine residue at position B22. The products obtained happen to be the first insulin derivatives described that contain a modified arginine residue in an otherwise unaltered molecule. As such they are of interest in clarifying the role of this particular residue, and it is this aspect that is discussed below.

Arg-B22 has been thought to be important to insulin action for several reasons, not least being the degree of genetic invariance; arginine is found at position B22 in all but two of 34 insulin sequences so far established (Dayhoff, 1972–1978; Horuk, 1979; Horuk *et al.*, 1980). The basic side chain of Arg-B22 is known to form a salt bridge with the C-terminus of the A chain in the crystal (Blundell *et al.*, 1972). Guinea-pig insulin exhibits an abnormally low activity in several standard bioassays, and it has been suggested that the substitution of aspartic acid

for arginine at position B22 may be responsible (Zimmerman *et al.*, 1974). Porcupine insulin, only recently characterized, is the other exception. It, too, is of low activity, and this has also been attributed to the substitution of aspartic acid for arginine at position B22 (Horuk *et al.*, 1980). There are, however, many other differences between these hystricomorph insulins and pig insulin. Support for the importance of Arg-B22 comes from chemical modification of insulin with phenylglyoxal (Bunzli & Bosshard, 1971). This reagent reacts with arginine residues and leads to an inactive product. However, the reagent is not specific: the α -amino groups were also modified. More recently, Weitzel *et al.* (1977) have replaced Arg-B22 with lysine, ornithine, citrulline, alanine and glycine in a series of despentapeptide-B26–B30-insulins and concluded that Arg-B22 is essential for insulin activity in these truncated analogues. The only result that might lead to a different conclusion is that obtained by Chu *et al.* (1980), who report that the truncated molecule despentapeptide-B26–B30-insulin with aspartic acid at position B22 is about as active in the mouse convulsion assay as is the despentapeptide-B26–30-insulin with arginine at position B22 (30–40% and 30% of the value for insulin respectively).

Cyclohexane-1,2-dione has been shown to react specifically with the arginine residues of proteins (Patthy & Smith, 1975*a,b*; Dietl & Tschesche, 1976; Smith, 1977) to produce a *cis*-diol moiety that is stabilized at alkaline pH by borate ion. In the absence of borate, arginine residues are slowly regenerated at neutral to alkaline pH (e.g. 10% in

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24 h at 40°C in phosphate buffer, pH 8.0), a process that is accelerated by nucleophiles (Patthy & Smith, 1975a). A different specifically modified derivative may be formed by periodate cleavage of the diol. This produces N^8N^9 -adipoylarginine residues which also slowly regenerate arginine residues under neutral to alkaline conditions. Pig insulin was therefore made to react with cyclohexane-1,2-dione. The product, dihydroxycyclohexylene-insulin {[N^8N^9 -(1,2-dihydroxycyclohex-1,2-ylene-arginine-B22)]insulin; DHCH-insulin in the nomenclature introduced by Patthy & Smith (1975a)}, was examined by electrophoresis on cellulose acetate and by amino acid analysis, and was shown to have been modified. Fig. 1 shows dihydroxycyclohexylene-insulin running faster than unmodified insulin towards the anode. This faster, running is due to complex-formation between borate

Fig. 1. Electrophoretograms of the modified insulins and native insulin

Experimental details are given in the text. FF shows the position reached by the dye Cyanol FF, applied close to the edge of the strip at the origin (O). (a) Electrophoretograms of (i) native pig insulin, (ii) adipoyl-insulin and (iii) dihydroxycyclohexylene-insulin, after incubation (5 mg/ml) in 33 mM-sodium phosphate buffer, pH 7.8, for a total of 20 h at 4°C and 3 h at room temperature. (b) Electrophoretograms of supernatants after incubation of (i) dihydroxycyclohexylene-insulin, (ii) native pig insulin and (iii) adipoyl-insulin (5 mg/ml) with the membrane preparation. The streaking seems to be due to the presence of bovine serum albumin, which was added (2 mg/ml) to the membrane preparation. It is clear that no great degree of regeneration of native insulin occurs.

and the *cis*-diol of the dihydroxycyclohexylene group. As expected, in the absence of borate (in phosphate/urea buffer, pH 7.2) no separation between dihydroxycyclohexylene-insulin and unmodified insulin could be achieved. Amino acid analysis, after hydrolysis in the presence of mercaptoacetic acid (Patthy & Smith, 1975a), indicated that the extent of modification of Arg-B22 was about 83%. That this is a minimum value for the extent of modification is apparent from the fact that there is little unmodified insulin visible on the electrophoretogram of the insulin derivative [Fig. 1a (iii)]. Cleavage of the dihydroxycyclohexylene group was performed and the product, [adipoyl-Arg-B22]insulin, was examined in the same way. At pH 8 in the borate/urea buffer, adipoyl-insulin runs slightly faster than dihydroxycyclohexylene-insulin (Fig. 1), which is consistent with the expected pK_a values of the adipoyl group and of the *cis*-diol-borate complex (between 4 and 5 and between 8 and 9 respectively). In the absence of borate (phosphate/urea buffer) a good separation between adipoyl-insulin and dihydroxycyclohexylene-insulin was achieved. Samples of the two modified insulins and unmodified insulin were administered to rats, and the blood glucose concentration was measured over a period of 4 h. There was no significant difference between the results obtained (Fig. 2), in spite of the fact that these derivatives have bulky substituents (six carbon atoms) covalently attached to the side chain of Arg-B22. In the case of the adipoyl derivative a negative charge is also introduced.

The surprising result that activity is retained in such derivatives could arise either because they are intrinsically as active as native insulin, or because they regenerate native insulin *in vivo* very much more rapidly than they do *in vitro* (or both of these). On examination of the membrane-binding characteristics of the derivatives, they were found to be almost as potent as native insulin during competition with radio-iodinated pig insulin bound to placental microvillar membranes (Rees & Whittle, 1980). (The total concentration of insulin at which half-maximal competition of labelled insulin was observed was 11 ng/ml, 10 ng/ml and 9 ng/ml for dihydroxycyclohexylene-insulin, adipoyl-insulin and unmodified insulin respectively.) Incubation of the derivatives with the binding assay buffer in the absence (Fig. 1a) and in the presence (Fig. 1b) of membranes did not give rise to any appreciable regeneration of native insulin. We therefore ascribe the binding activity, and possibly the early part of the result obtained *in vivo*, to the intrinsic insulin-like activity of these derivatives modified at Arg-B22. These findings lend support to the suggestion (Chu *et al.*, 1980) that the low activity of guinea-pig insulin might be due to some change in the molecule other than the substitution of aspartic acid for

Fig. 2. Blood glucose concentration as a function of time after administration of the modified insulins and native insulin

Experimental details are given in the text. (a) Blood glucose concentration after administration of dihydroxycyclohexylene-insulin ($10.7 \mu\text{g/kg}$) [O and vertical bar, mean and S.E.M. (one bound, $n = 7$)] or of native insulin ($10.7 \mu\text{g/kg}$; 0.25 unit/kg) [● and vertical bar, mean and S.E.M. (one bound, $n = 5$)]. (b) Blood glucose concentration after administration of adipoyl-insulin ($60 \mu\text{g/kg}$) [vertical bars, mean and extrema (of 3)] or of native insulin ($63 \mu\text{g/kg}$; 1.5 units/kg) (O, values for 2 rats).

arginine at position B22. Such a conclusion is consistent with results obtained by L. Newman & A. R. Rees (unpublished work), who have prepared hybrid insulins from purified A and B chains of guinea-pig and pig insulin and who have shown that the hybrid guinea-pig A/pig B insulin, which retains the arginine residue at position B22, has essentially no binding activity and is inactive in biological assays. It thus seems clear that the intrinsically low activity of guinea-pig insulin is not simply due to the substitution at position B22. A possible explanation, which would also account for the observed high degree of genetic conservation of this residue and for

the salt bridge, would be that Arg-B22 is important during the folding of proinsulin to the native conformation. After the folding and subsequent conversion into insulin by cleavage of the C-peptide, the stability of the folded insulin molecule would then be sufficient to permit even quite drastic changes in the nature of the arginine side chain without affecting the overall conformation of the binding site.

Experimental

Dihydroxycyclohexylene-insulin was prepared by treating pig insulin (10 mg/ml) with cyclohexane-1,2-dione (0.15 M) in 8 M -urea in 0.25 M -sodium borate buffer, pH 9, at 37°C for 2 h, then adding an equal volume of cold 30% (v/v) acetic acid and dialysing the mixture exhaustively at 4°C against 1% acetic acid. Adipoyl-insulin was prepared by treating dihydroxycyclohexylene-insulin (58 mg) for 20 h at 4°C in the dark with sodium metaperiodate (2.25 mg) in 11 ml of 8 M -urea in 0.2 M -sodium acetate adjusted to pH 4.0 with 1 M -HCl. The product was isolated by extensive dialysis against 1% acetic acid and was freeze-dried. [Polypeptides containing arginine residues modified with cyclohexane-1,2-dione, such as dihydroxycyclohexylene-insulin, may be freed from any unmodified material by chromatography on a column of immobilized borate (Patthy *et al.*, 1979; Rose, 1979). The same approach could be used to remove traces of unchanged dihydroxycyclohexylene-insulin from preparations of adipoyl-insulin.]

For electrophoresis strips of cellulose acetate 3 cm wide were used, the voltage gradient being $200 \text{ V}/15 \text{ cm}$. The buffer was 6.4 M -urea in 0.2 M -sodium borate buffer, pH 8.0. Staining was effected with 0.2% Ponceau S in 3% acetic acid, and destaining with 3% acetic acid.

Insulin or derivative was administered by intravenous injection to anaesthetized rats that had been deprived of food for 24 h. At set times, approx. 0.25 ml of blood was drawn for analysis, via a polythene catheter inserted in a jugular vein, and replaced by 0.9% NaCl containing 50 i.u. of heparin/ml. The glucose analyses were performed by the glucose oxidase/periodate method according to the instructions of LKB Produkter, Bromma, Sweden.

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