The close juxtaposition of residues A1 and B30 in the insulin structure shows that only a few residues of the rather long and species-variable connecting peptide of proinsulin are required to connect these groups. The position of residues A1 and B30 on the surface of the hexamer is consistent with the reported dimerization and hexamerization of proinsulin.

Most of the species-variable residues are on the surface of the insulin hexamer, and have been implicated by studies on modified insulins in the binding of antibodies. In general, residues implicated in the same antibody-binding site occupy adjacent positions on the surface of the insulin molecule.

Residues in a region of the hexamer surface, including glycine-A1, glutamine-A5, tyrosine-A19 and residue B25 (aromatic amino acid), are invariant. Chemical and spectral studies indicate that modification of some of these residues usually gives rise to changes of conformation and aggregation of the insulin molecules. Further, the biological activity is extremely sensitive to these modifications. In particular, the striking loss of biological activity associated with deamination of residue A1 or removal of residue A21 may imply that subtle changes of conformation on binding are an integral part of the insulin mechanism. Until reliable information about the state of aggregation of the active species is obtained it is impossible to say whether the invariant non-polar residues such as valine-B12 and phenylalanine-B24 that are involved in dimerization are also important to the activity. If so, the region involved in binding to the receptor may include all these groups. In this case the interactions would be largely hydrophobic, perhaps with hydrogen bonds involving the peptide groups of residues B24 and B26 (usually involved in the antiparallel sheet structure of the dimer). Whether the mechanism of insulin action at the receptor is simply a 'lock-and-key' fit or a more subtle induced fit involving changes of structure of the insulin molecule and receptor, the initial integrity of the general tertiary structure of much of the insulin molecule, and certainly this complex region, appears to be necessary for activity.

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Semisynthetic Insulin Analogues

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One approach to the preparation of insulin analogues is to start with insulin and to carry out a partial degradation (e.g. the preparation of des-B30-alanine-des-A21-asparagine-insulin; Nicol, 1960; Slobin & Carpenter, 1963) or to effect chain lengthening by peptide synthesis, yielding aminoacyl-insulins (Levy & Carpenter, 1966, 1967).

One other possibility is the transformation of the isolated insulin chains by partial degradation or partial synthesis into chain analogues that are subsequently combined to give insulin analogues. Bovine insulin A-chain-S-sulphonate was treated with phenyl isothiocyanate and the resulting phenylthiocarbamoyl derivative treated with trifluoroacetic acid to remove the *N*-terminal glycine residue. The des-glycine-A-chain-S-sulphonate contained less than 2% of glycine and was combined with bovine insulin B-chain-S-sulphonate by following the procedure of Du, Yang & Tsou (1965). The electrophoretically homogeneous des-A1-glycine-insulin, obtained on gel filtration [Sephadex G-50 (fine grade), with 0.05M ammonium bicarbonate pH 8.2] and ion-exchange chromatography (CM-Sephadex, with 0.5-5M-acetic acid gradient), gave the expected amino acid analysis. It did not crystallize, and the biological activity as determined in the isolated-fat-cell assay was 2-10% (Gliemann, 1965).

The immunoreactivity in the double-antibody radioimmunoassay (Morgan, Sorenson & Lazarow, 1964) was 20-30% as compared with that of bovine insulin at the point of 50% displacement of labelled insulin from the antibodies. Des-glycine-A-chain-S-sulphonate was also used for reaction with *p*-nitrophenyl [¹⁴C]acetate. The acetylated chain was isolated by gel filtration (at pH 8.2) and ion-exchange chromatography (Shimonishi & Zahn, 1968), and combined with B-chain-S-sulphonate to yield non-crystalline des-A1-amino-insulin, showing 15% of insulin's biological activity and 40% of its immunoreactivity. Crystalline A0-arginyl-insulin (Weinert, 1971; Weinert, Kircher, Brandenburg & Zahn, 1971) with 59% (mouse-convulsion assay) and 68% (fat-pad assay) biological activity was prepared starting with A-chain-S-sulphonate, which was acylated with the *N*-carboxyanhydride of arginine hydrobromide. [Shimonishi (1970) has also reported on the preparation of semisynthetic insulin A-chain-S-sulphonates.] The resulting

arginyl-A-chain-S-sulphonate was isolated in pure form by chromatography on DEAE-Sephadex and combined with bovine insulin B-chain-S-sulphonate. From the combination product 15% of arginyl-insulin was isolated. The crystallization from citrate buffer, pH 7, containing Zn^{2+} was nearly quantitative. A-1-lysyl-A0-arginyl-insulin, a non-crystallizable analogue that contains the last two amino acid residues of the proinsulin C-chain, had a biological activity of 20% and an immunoreactivity of 34%. It was prepared by chain lengthening of arginyl-A-chain-S-sulphonate with bisbutyloxycarbonyl-lysine trichlorophenyl ester, removal of the protecting groups by treatment with trifluoroacetic acid and combination of the isolated lysyl-arginyl-A-chain-S-sulphonate with B-chain-S-sulphonate.

The bovine insulin B-chain-S-sulphonate was also subjected to partial degradation in experiments similar to those described for the A-chain. One cycle of preparative Edman degradation yielded des-B1-phenylalanine-B-chain-S-sulphonate, from which a second cycle gave des-B1-phenylalanine-des-B2-valine-B-chain-S-sulphonate, characterized by electrophoresis and amino acid analysis (Herbertz, 1971).

For the preparation of [B1-*p*-iodophenylalanine]-insulin, two routes were followed. Both des-B1-phenylalanine-B-chain-S-sulphonate (route 1) as well as A1,B29-bisbutyloxycarbonyl-des-B1-phenylalanine-insulin (kindly given to us by Dr G. Geiger, Farbwerke Hoechst AG, Frankfurt-Hoechst, Germany) (route 2) were acylated with butyloxy-carbonyl-*p*-iodophenylalanine trichlorophenyl ester, the latter in dimethyl sulfoxide as solvent and *N*-methylmorpholine as a base. Cross-linking reactions (Brandenburg, 1971) were not observed. Deblocking and purification of the insulin derivative (method 2) gave [B1-*p*-iodophenylalanine]-insulin, which crystallized as spindles and possessed a biological activity of 60–70%.

The circular-dichroism spectra of all analogues (A. Wollmer, unpublished work) are significantly different from that of insulin, indicating that the loss of biological activity is due to changes of the three-dimensional structure of the molecule.

In conclusion, semisynthetic studies in the field of insulin chemistry look promising not only with transformations of the two-chain molecule but also with peptide synthesis involving the separated chains followed by chain combination. This latter operation seems not to be hampered by the fact that modified chains are combined.

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The Structure of Insulin in Relation to its Immunological Activity

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The identification of surface conformations on insulin to which antibodies bind is important in defining the interactions of insulin with other macromolecules. By comparing the immunologic activity of derivatives of insulin in the immune-haemolysis inhibition assay and in a radioimmune assay it has been possible to distinguish the determinants on insulin that are detected by the two assays. The elucidation of the secondary and tertiary structure of insulin (Blundell, Dodson, Dodson, Hodgkin & Vijayan, 1971) has permitted a more precise definition of such surface conformations.

The immunological studies concerned with the structure-function relationships of the insulin molecule that have been reported were concerned primarily with the use of insulin antisera prepared in strain 2 and strain 13 inbred guinea pigs. These antisera were tested for their ability to react with various derivatives of insulin containing known alterations in primary structure (Arquilla, Bromer & Mercola, 1969). These studies demonstrated that strain 2 antibodies react with determinants involving the free amino groups of insulin, whereas the strain 13 antibodies reacted preferentially with determinants involving the C-terminal portions of the A- and B-chains. They also indicated that insulin has a very tightly packed core, rich in