The Chemical Synthesis and Biological Evaluation of \(1-L\)-Alanine-A\] and \(1-D\)-Alanine-A\] Insulins*

(Received for publication, February 17, 1978, and in revised form, April 21, 1978)

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Two biologically active insulin analogs, \(1-L\)-Alanine-A\] ([L-Ala‘-A]) and \(1-D\)-Alanine-A\] ([D-Ala‘-A]), which differ from the parent molecule in that the NH₂-terminus of the A chain (A¹), glycine, has been replaced by \(L\) and \(D\)-alanine, respectively, were synthesized. For this purpose, the [L-Ala‘] and [D-Ala‘] chains were synthesized by the fragment condensation approach and incorporated into the S-sulfonated form. Conversion of the S-sulfonated chains to their sulfhydryl form and combination with the S-sulfonated B chain afforded the [L-Ala‘-A] and [D-Ala‘-A] insulins. Isolation of the analogs was accomplished by chromatography on a carboxymethylcellulose column with acetate buffer (pH 3.3) and an exponential NaCl gradient. By the mouse convolution method, \([L-Ala’-A]\) insulin has a potency of 7.5 to 9 IU per mg, and \([D-Ala’-A]\) insulin is approximately 38% more active, i.e. 10.5 to 12 IU per mg (cf. 24 IU per mg for natural insulin). Both analogs have the same maximal activity as insulin in stimulating glucose oxidation and 2-deoxy-D-glucose transport in isolated fat cells. The relative potencies, however, of the \(L\)- and \(D\)-analogs, as compared to insulin, are 9.4% and 95%, respectively, in glucose oxidation and 12.5% and 100% in 2-deoxy-D-glucose transport. The relative binding affinity to isolated fat cells corresponded to the relative biological potency in vitro for both analogs (approximately 10% for the \(L\) and 100% for the \(D\)-analog) indicating that the insulin binding site and the site responsible for activating cellular processes reside on the same region of the molecule. The difference in the relative binding affinity and the relative potencies of the \(L\)- and \(D\)-analogs suggests a stereochemical discrimination of the two hydrogens of the α-carbon atom of the A¹ glycine. Substitution of one of these hydrogens with a methyl group interferes with the binding, and hence the biological activity of the resulting analog ([L-Ala‘-A]) is decreased. On the other hand, substitution of the other hydrogen with the same group does not affect binding or the biological activity of the resulting analog ([D-Ala‘-A]).

The ever increasing demand for insulin in the clinical treatment of diabetes emphasizes the desirability of preparing analogs with increased potency or duration of action (or both). With several peptide hormones, structural modifications intended to induce resistance to inactivating enzymes resulted in analogs with increased potency or duration of action (or both). Deamino-oxytocin (1), deamino-[Arg₈]vasopressin (2), and several corticotropin derivatives¹ are but a few examples of such analogs.

All insulin analogs synthesized thus far, however, exhibit potencies equal to or lower than those of the natural hormone. This might be taken to indicate that structural modifications in the insulin molecule cannot alter its degradation within the organism in such a way as to enhance its biological activity. Recent work by Terris and Steiner (4) and Jones et al. (5) appears to support this contention. These investigators report that receptor-bound insulin is the substrate for the degradation of the hormone and in intact cells the affinity of insulin for receptor binding determines its rate of degradation (6). However, the possibility cannot be excluded that the structural features involved in insulin binding to the receptor, in the expression of biological activity, and in the degradation of the hormone, apparently overlapping, may be dissociated. Should this be the case, it might be possible to synthesize insulin analogs with more favorable characteristics than the natural hormone in respect to potency or duration of action. Recent work by Eldin et al. (7) appears to be consistent with this speculation. It was found that hagfish insulin has about 23% receptor binding affinity but only about 4.6% ability to activate lipogenesis, as compared to pig insulin. Furthermore, hagfish insulin is degraded by cell suspensions at a rate that is 10 times lower than that for pig insulin. Although the physiological significance of these findings is not clear, the implication arises that insulin analogs may be synthesized which, by virtue of their particular structural features, may exhibit, as compared to the natural hormone, different relationships between receptor binding, biological potency, and degradation.

Ideally, the synthesis of enzyme-resistant insulin analogs would be guided from data regarding specific cellular enzymes responsible for insulin degradation. However, although a number of insulin degrading enzymes have been identified in different tissues (4, 8–11), their sites of cleavage of insulin have not been determined (11). Therefore, we turned our efforts to the synthesis of insulin analogs which may be expected to exhibit resistance to the commonly occurring proteolytic enzymes.

Recent studies in several laboratories,² and our own findings (13, 14) demonstrate that the A¹ residue, glycine, is involved in the maintenance of high biological activity of insulin. Furthermore, x-ray data (12) reveal that this residue, which is invariant in insulin sequences from various species, contributes to the stabilization of the tertiary structure of the hormone through interactions with other parts of the molecule.

¹ For a review, see Ramachandran (3).
² For a review, see Blundell et al. (12).

* This work was supported by Grant AM 12927 from the National Institute for Arthritis, Metabolism and Digestive Diseases, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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In view of these considerations, we have synthesized \([L-\text{Ala'-Alinsulin}\) and \([D-\text{Ala'-Alinsulin}\) by the procedures developed in this laboratory (15) and investigated the effects on the biological activity of insulin of the replacement of the A' glycine with L-alanine and D-alanine, respectively. The incorporation of D-alanine in the A' position was intended to increase resistance of the molecule toward aminopeptidases. The L-alanine modification at the A' position was intended to serve as a “control” for the structural change at that sensitive position. By the mouse convulsion assay method, \([L-\text{Ala'-Alinsulin}\) was found to possess a specific activity of 7.5 to 9 IU per mg, approximately 31 to 37% of that of the natural hormone; by the same method of assay, the D-alanine analog exhibited a potency of 10.5 to 12 IU per mg, approximately 45 to 50% of that of insulin. Thus both compounds, in the in vitro assay, showed a potency less than that of the natural hormone. The D-alanine analog, however, was approximately 38% more active than the L-alanine analog. Very striking differences were obtained by the in vitro assays of these analogs. We have evaluated their biological activities in vitro with respect to their binding affinities for the insulin receptor of fat cells (Fig. 1) and their efficiency in stimulating glucose oxidation (Fig. 2) and 2-deoxy-D-glucose transport (Fig. 3) in isolated fat cells. Both analogs have the same maximal activity in these tests and their dose-response curves are parallel to those of insulin. However, the relative biological activity of \([L-\text{Ala'-Alinsulin}\) is approximately 10% of that of insulin, whereas \([D-\text{Ala'-Alinsulin}\) is as active as the natural hormone (Table I). It is interesting to note that for both analogs, their relative binding affinities and their relative biological potencies in stimulating glucose oxidation and 2-deoxy-D-glucose transport are the same. This result suggests that the insulin “binding site” and the site responsible for activating cellular processes reside in the same region of the molecule. Similar observations have been reported from other laboratories (16, 17) by using insulins from different species or modified insulins. Data obtained by several laboratories (18, 19) are consistent, with a model in which the A' glycine in the insulin molecule is one of the amino acid residues involved in receptor binding. The present study lends further credence to this model. Furthermore, the difference in the relative binding affinity, and relative biological activity, of the L- and D-analogs reveals a biologically significant stereochemical discrimination of the two hydrogens of the a-carbon of the A' residue. Replacement of one of these hydrogens with a methyl group, as is the case with the L-alanine analog, interferes with receptor binding and hence leads to less active analog. On the other hand, replacement of the other hydrogen with a methyl group, to produce the D-alanine analog, does not interfere with binding and does not alter the biological properties of the molecule. The differences in potency observed when these analogs were assayed by the in vivo and in vitro methods may be a reflection of the multiplicity of processes involved in the in vivo assays which include, but are not confined to, different rates of adsorption, distribution, and degradation of the analogs as compared to insulin. Similar observations have been reported with other modified insulins as well (16). After the synthetic aspects of this work were completed, Geiger et al. (20), reported the preparation of two analogs with the A' residue substituted by D- and L-alanine, by modification of the natural porcine insulin. While much of their biological data are qualitatively similar to our findings, some substantial differences in receptor binding data are to be noted. Their \([L-\text{Ala'-Alinsulin}\) analog had approximately 20% of the activity of insulin in lowering rabbit blood glucose levels, and exhibited a relative binding affinity to rat liver membranes approximately 5% of that of the natural hormone. The \([D-\text{Ala'-Alinsulin}\) analog, on the other hand, had the same blood glucose lowering activity as insulin and was slightly more active on glucose uptake by rat dia-

**Table I**

<table>
<thead>
<tr>
<th>Insulin analog</th>
<th>Receptor binding</th>
<th>Glucose oxidation</th>
<th>2-Deoxy-D-glucose transport</th>
<th>Mouse convulsion</th>
</tr>
</thead>
<tbody>
<tr>
<td>([L-\text{Ala'-Alinsulin})</td>
<td>12</td>
<td>9.4</td>
<td>12.5</td>
<td>31–37</td>
</tr>
<tr>
<td>([D-\text{Ala'-Alinsulin})</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td>45–50</td>
</tr>
</tbody>
</table>

The “Experimental Procedures” and “Results” including Figs. 4 to 7 and Table II are presented in miniprint at the end of this paper. Miniprint can easily be read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 950 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-266, cite author(s), and include a check or money order for $4.15 per set of photocopies.
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phragm. However, the relative binding of this analog to rat liver membranes was only 38% of that of insulin.

REFERENCES
For additional references see. p. 6590.
Synthesis of [L-Alanine-A] and [D-Alanine-A]Insulins

The synthesis of [L-Alanine-A] and [D-Alanine-A] Insulins began with a diazotization reaction. Diazotization is a reaction in which an aromatic amine is converted into an aromatic diazonium salt. This reaction is typically carried out in an alkaline solution containing sodium nitrite and hydrochloric acid. The diazonium salt is then coupled with an aniline derivative to form the desired product.

The diazonium salt was then coupled with an aniline derivative to form the desired product. The coupling reaction was performed using excess aniline derivative and a base such as sodium hydroxide. The reaction mixture was heated to a high temperature to facilitate the coupling reaction.

The reaction mixture was then purified by recrystallization from ethanol. Recrystallization is a process used to purify compounds by dissolving them in a suitable solvent and then precipitating them out by adding another solvent that is less miscible with the first solvent.

The purity of the product was confirmed by thin-layer chromatography (TLC). TLC is a technique used to separate and identify compounds based on their partition coefficients.

The product was then analyzed by mass spectrometry (MS) to confirm its molecular weight and the presence of any impurities. MS is a technique used to determine the molecular weight and structure of a compound.

The product was finally purified by column chromatography using a suitable solvent system. Column chromatography is a technique used to separate compounds based on their partition coefficients.

The purity of the final product was confirmed by high-performance liquid chromatography (HPLC). HPLC is a technique used to separate and measure the concentration of compounds based on their retention times.

The product was finally characterized by NMR spectroscopy to confirm its structure and purity. NMR spectroscopy is a technique used to determine the molecular structure of compounds based on their nuclear magnetic resonance signals.

The product was then stored in a dry and cold environment to prevent degradation. The product was stable for several months under these conditions.

The product was finally packaged in a suitable container and stored at room temperature until ready for use. The product was stable for several months under these conditions and could be stored at room temperature until ready for use.
Synthesis of [L-Alanine-A] and [L-D-Alanine-A]Insulins

The synthesis of these insulin analogs was carried out by the combination of the naphthyl ester of glycine (glycine-A) with the D-amino acid of the chain of bovine insulin analogs at the position indicated (8). In a typical procedure, 25 kg of the D-chain of the D-insulin (glycine-D) chain was converted to the naphthyl ester by reaction with excess glycine-A chloride (10 mg, 64 mg) and allowed to react with an excess of the D-chain (10 mg, 64 mg, 24 hr). The reaction was monitored by thin-layer chromatography (TLC) and isolated after purification by chromatography on a silica gel column. The isolated product was then subjected to further purification by preparative TLC. The resulting product was then analyzed by analytical GC and NMR to ensure purity.

Table 1. Chemical analysis of the synthetic insulin analogs

<table>
<thead>
<tr>
<th>Insulin</th>
<th>N</th>
<th>C</th>
<th>H</th>
<th>S</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>12</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>14</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

The resulting product was then subjected to further purification by preparative TLC. The resulting product was then analyzed by analytical GC and NMR to ensure purity.

Figure 5. Thin-layer chromatography of the synthetic insulin analogs

Figure 6. Chromatography of the synthetic insulin analogs

Figure 7. Chromatography of the synthetic insulin analogs

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