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)

Alexandros Cosmatos, Kang Cheng, Yoshio Okada, and Panayotis G. Katsoyannis†

From the Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

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([(D-Ala']A)), which differ from the parent molecule in that the NH₂-terminus of the A chain (A'), glycine, has been replaced by L- and D-alanine, respectively, were synthesized. For this purpose, the [L-Ala']A and [D-Ala']A chains were synthesized by the fragment condensation approach and isolated in 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 carboxymethyl cellulose 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-o-glucose transport in isolated fat cells. The relative potencies, however, of the L- and D-analogs, as compared to insulin, are 94% and 95%, respectively, in glucose oxidation and 12.5% and 100% in 2-deoxy-o-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 A' 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). Deaminoxytocin (1), deamine(D-Arg)vasopressin (2), and several corticotropin derivatives are 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 Emdin 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, and our own findings (13, 14) demonstrate that the A' 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.

* 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.

† To whom correspondence should be addressed.
In view of these considerations, we have synthesized \([\text{L-Ala'}]^{-}\) insulin and \([\text{D-Ala'}]^{-}\) insulin 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, \([\text{L-Ala'}]^{-}\) insulin 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 \(in\ v\itro\) 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 \(in\ v\itro\) assays of these analogs. We have evaluated their biological activities \(in\ v\itro\) 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 \([\text{L-Ala'}]^{-}\) insulin is approximately 10% of that of insulin, whereas \([\text{D-Ala'}]^{-}\) insulin is as active as the natural hormone (Table 1). 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 \(D\)-analogues reveals a biologically significant stereochemical discrimination of the two hydrogens of the \(\alpha\)-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 \(in\ v\itro\) and \(in\ v\itro\) methods may be a reflection of the multiplicity of processes involved in the \(in\ v\itro\) 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

\[ \text{Table I} \]

<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>([\text{L-Ala'}^{-}]^{-}) insulin</td>
<td>12</td>
<td>9.4</td>
<td>12.5</td>
<td>31-37</td>
</tr>
<tr>
<td>([\text{D-Ala'}^{-}]^{-}) insulin</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td>45-50</td>
</tr>
</tbody>
</table>

similar to our findings, some substantial differences in receptor binding data are to be noted. Their \([\text{L-Ala'}^{-}]^{-}\) insulin 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 \([\text{D-Ala'}^{-}]^{-}\) insulin, on the other hand, had the same blood glucose lowering activity as insulin and was slightly more active on glucose uptake by rat dia-
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 \([l\text{-Alanine-A}]\) and \([l\text{-D-Alanine-A}]\) Insulins

In conclusion, it was observed that...

*Note: The text is a transcription of a scientific abstract and may require context to fully understand its implications.*
Synthesis of [l-L-Alanine-A] and [l-D-Alanine-A]Insulins

A Cosmatos, K Cheng, Y Okada and P G Katsoyannis