Structure and Function Relationships of Insulin

PREPARATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY OF THREE BOVINE INSULIN DERIVATIVES SELECTIVELY MODIFIED AT THE NH$_2$-TERMINAL OF THE B CHAIN*

(Received for publication, March 5, 1979, and in revised form, May 24, 1979)

Clement W. T. Yeung,† Margaret L. Moule, and Cecil C. Yip§

From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada MSG 1L6

There have been extensive studies on the selective modification of the amino groups of insulin (2-4) and of the NH$_2$-terminal of the B chain. Removal of the NH$_2$-terminal B1 phenylalanine does not lead to any changes in biological activities (5, 6), although reduced immunological reactivities was observed with des-(Phe-Val-Asn)$\beta$-p(Glu)$\beta$4-insulin (8, 11). In the present study, we wish to extend these studies and further investigate the structure, conformation, and activity relationships of the NH$_2$-terminal region of the B chain of bovine insulin. Our approach was to perturb the region around the B1 phenylalanine terminus by the addition of an amino acid with hydrophobic or hydrophilic side chain and to study the effects of such perturbation on the conformation and biological activities of this hormone. The functional importance of this region in contributing, directly or indirectly, to the biological activities of insulin could then be assessed. In view of these considerations, we have semisynthesized three insulin derivatives, N$\beta$1(L-methionyl)insulin, N$\beta$1(L-lysyl)insulin, and N$\beta$1(L-arginyl)insulin, and studied their biological activity, receptor binding activity, immunoreactivity, and conformation.

The in vitro biological activities and the receptor binding affinity of the analogues appear to correlate well with each other (Table I). The lower biological potency of the derivatives is attributable to their corresponding lower affinity for the plasma membrane insulin receptor. In the present study, using both in vitro bioassay and receptor binding assay, the biological activity of N$\beta$1(L-lysyl)insulin was found to be much higher than the activity of 41 ± 2% for this analogue as reported by Krahl et al. (11). The cause of this discrepancy is not known. It is of interest to find that N$\beta$1(L-lysyl)insulin and N$\beta$1(L-arginyllnsulin are significantly different in their biological activity, even though the side chain in both derivatives is positively charged and hydrophilic in nature. Structurally, the side chain of lysine may offer greater flexibility than the side chain of arginine. It may be that the flexibility of the side chain of lysine at the NH$_2$-terminal of the B chain allows a better interaction with the insulin receptor resulting in a higher biological activity than N$\beta$1(L-arginyl)insulin. The general decrease in the biological activity of the derivatives compared to bovine insulin may be the result of structural changes. Although removal of the B1 phenylalanine does not cause changes in the biological activity of insulin, it is possible that the addition of amino acid residues on the NH$_2$-terminal of the B chain may lead to local structural changes which induce secondary and/or tertiary structural changes. These

* This work was supported by grants from the Medical Research Council, Canada and the C. H. Best Foundation. A preliminary account of this work was presented previously (1). 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.

† Recipient of the C. H. Best Postdoctoral Fellowship.

§ To whom reprint requests should be sent.
TABLE I

Biological activities of bovine insulin derivatives

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Potency IU/mg</th>
<th>95% Confidence Limit</th>
<th>Index of precision (A)</th>
<th>% (Range)</th>
<th>Membrane binding</th>
<th>Radioimmunoassay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine insulin*</td>
<td>26.42</td>
<td>24.81-28.15</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>N(^{ab})(L-Methionyl)insulin</td>
<td>16.77</td>
<td>13.67-20.62</td>
<td>0.11</td>
<td>63.5 (62-68)</td>
<td>54</td>
<td>65</td>
</tr>
<tr>
<td>N(^{ab})(L-Arginyl)insulin</td>
<td>10.15</td>
<td>7.95-12.94</td>
<td>0.08</td>
<td>38.4 (39-40)</td>
<td>43</td>
<td>40</td>
</tr>
<tr>
<td>N(^{ab})(L-Lysyl)insulin</td>
<td>20.68</td>
<td>18.94-22.58</td>
<td>0.09</td>
<td>78.4 (78-82)</td>
<td>69</td>
<td>58</td>
</tr>
</tbody>
</table>

*Bovine insulin (lot 1053) supplied by Connaught Medical Research Laboratories, Toronto, Ontario, Canada.

**Changes** may not be great enough to cause an overall conformational change in the molecule, but they may either directly affect the interaction of the derivatives with the insulin receptor or be transmitted toward other regions (12, 13) of the insulin molecule whose structural integrity is deemed essential in the receptor binding.

CD studies of insulin have shown that the major contribution to the negative ellipticity at 273 nm comes from the aromatic residues (tyrosyl and phenylalanyl) and their optical activities are a function of aggregation of the molecule (14, 15). The weakening of the 273-nm band seen with N\(^{ab}\)(L-methionyl)insulin, N\(^{ab}\)(L-lysyl)insulin, and N\(^{ab}\)(L-arginyl)insulin (Fig. 1a) may, therefore, be attributed to the decrease in association of the corresponding monomeric insulin species. In the 190- to 250-nm region of the CD spectra, the 222-nm band is assigned to \(\alpha\) structure (16), which is a predominant feature of the dimer molecule of insulin. The attenuation in this particular band observed in the three derivatives showed a decrease in \(\beta\) structure probably as a result of decrease in dimer formation, especially in the case of N\(^{ab}\)(L-arginyl)insulin (Fig. 1b). The 208-nm band is assigned to the \(\alpha\) helix structure (17). X-ray studies of insulin (2) show that major contribution to \(\alpha\) helix structure comes from residues B10 to 19 with additional contributions from A2 to 6 and A13 to 19. The ratio of \([\theta]_{208}/[\theta]_{222}\) was 1.35 in both N\(^{ab}\)(L-methionyl)insulin and N\(^{ab}\)(L-lysyl)insulin and differed only slightly from a value of 1.33 for bovine insulin. However, this ratio was markedly higher in N\(^{ab}\)(L-arginyl)insulin and, together with the decrease in the shoulder at 222 nm, it suggests that the \(\beta\) structure in this derivative is probably absent. In fact, the 190- to 250-nm region of the CD spectra of this derivative is comparable to that of guinea pig insulin as reported by Wood et al. (18). From their data, a ratio of \([\theta]_{208}/[\theta]_{222}\) for guinea pig insulin was estimated to be 1.75 compared with 1.16 for bovine insulin. A value of 1.69 for N\(^{ab}\)(L-arginyl)insulin was obtained in this study. Since guinea pig insulin is known to exist only as a monomer and does not show any concentration-dependent aggregation (19), our studies suggest that N\(^{ab}\)(L-arginyl)insulin may share this property.

The properties of the three semisynthetic insulin derivatives may be compared to several naturally occurring insulins with special reference to the alteration of the NH\(_2\)-terminal region of the B chain. Thus, N\(^{ab}\)(L-methionyl)insulin with a biolog-
ical activity of 16.77 IU/mg may be analogous to cod insulin and toadfish insulin, both of which have methionine as the NH$_2$ terminus at position B0 of the B chain and reduced activity of 11.5 IU/mg for cod insulin (20) and 7.7 IU/mg for toadfish insulin (21). However, in both the cod and the toadfish insulin, there are a total of 16 amino acid substitutions in the primary sequence compared with bovine insulin. Although the majority of these amino acid substitutions are considered conservative replacements, the tertiary structure of the respective insulin will likely reflect these primary structural differences and, thus, dictates its functional properties. Since conformational data on these insulin molecules are not available, it is impossible to assign functional importance to the NH$_2$-terminal of the B chain of these insulins. The other insulin derivative, N$_{\text{B1}}$-arginylinsulin, may be compared to the Atlantic hagfish (Myxine glutinosa) insulin whose NH$_2$ terminus of the B chain is arginine at position B1 (22) and whose biological activity is about 5% that of mammalian insulin (23, 24). Despite the fact that there are 19 amino acid replacements in the primary sequence of the Atlantic hagfish insulin, the low resolution (6 Å) x-ray crystallographic study (25) showed that the folding of the polypeptide backbone of the Atlantic hagfish insulin is similar to that of the pig insulin. This would suggest that the spatial position of the B1 arginine in the Atlantic hagfish insulin could be similar to that of the B1 phenylalanine in pig or bovine insulin. In the present study, introduction of an arginine residue at position B0 in bovine insulin led to a derivative having only 38% of biological activity. It might be possible that introduction of an arginine residue at position B1 in bovine insulin would lead to a further decrease in biological activity. In general, it is difficult to demonstrate the functional significance of the NH$_2$-terminal of the B chain; nevertheless, it is interesting to note that insulin of these species (with the NH$_2$-terminal amino acid of the B chain changed from R1 phenylalanine (an invariant in mammalian insulin) to B0 methionine (in cod and toadfish insulin) or B1 arginine (in Atlantic hagfish insulin) have much lower biological activities in comparison with most mammalian insulins.

In conclusion, the differences in the CD spectra detected in the insulin derivatives are attributable to the changes in their state of aggregation as a result of the addition of the new amino acid onto the NH$_2$-terminal of the B chain. The ability of the insulin molecule to associate to form $\beta$ structure and its ability to interact with the receptor are believed to be interrelated (12). In our studies the biological properties of the derivatives appear to show a dependence on their ability to associate since the decrease in both the biological potency and the membrane binding affinity parallels the degree of attenuation of the CD spectra.

Acknowledgement—We wish to thank Dr. David Kella, Department of Biochemistry, University of Toronto, for help with the CD studies.

REFERENCES
25. Additional references can be found on page 9457.
SUPPLEMENTAL MATERIAL TO

STRUCTURAL AND FUNCTION MODULATIONS OF INSULIN.

PREPARATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY OF THREE NOVEL INSULIN DERIVATIVES

SELECTIVELY MODIFIED AT THE N-TERMINUS OF THE B-CHAIN

GLENALY M. YANG, HONG CHEN, AND GEORGE C. TIP

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and Analytical Methods - Crystalline bovine zinc insulin was a gift from Ciba-Geigy. Paramount Crystallographic, Toronto, Canada. 82-Hoc-L-Arg-His (N, N-dipropyl) (L, L-Arg-His) was obtained from Sigma and Boc-L-Arg-His (N, N-dipropyl) (L, L-Arg-His) was prepared in the author's laboratory. Amino acids and reagents were purchased from Pierce Chemical Co., Rockford, Illinois. Antibiotics were obtained from Sigma Chemical Co. All other reagents and solvents were of analytical grade and were used without further purification. WAT, 82-Hoc-L-Arg-His (N, N-dipropyl)-L-Arg-His (L, L-Arg-His) was prepared according to the procedure described by Bear et al. (21) and was purified according to procedures outlined by Knapp et al. (11).

Semi-Synthetic Insulin Derivatives - The procedure used by Park et al. (22) was modified to include a purification step to reduce the final yield. The gel was eluted with 0.05 M Tris-HCl (pH 8.5) and was dialyzed against a solution containing 0.01 M Tris-HCl (pH 8.5) and 0.05 M NaCl. The gel was then neutralized with 50 mM CO2 and was dialyzed against a solution containing 0.01 M Tris-HCl (pH 7.0) and 0.05 M NaCl. The final insulin was dialyzed against a solution containing 0.01 M Tris-HCl (pH 8.5) and 0.05 M NaCl.

Protein Analysis - The analysis were carried out according to the procedure of Lowry et al. (23) with a bovine hemoglobin assay. Acetic acid-insoluble material was precipitated by 80% acetic acid. The samples were then centrifuged and the supernatant was removed. The remaining material was washed with 80% acetic acid and dried. The dry weight of the material was then determined.

In Vivo Bioassay - The biological activity of the insulin derivatires was determined using a standard method (24). Insulin derivatires were injected subcutaneously into adult rats and blood was collected at 60 min after injection. The blood was then analyzed for insulin.

RESULTS AND DISCUSSION - The results of the present study are summarized in Table I. The semi-synthetic insulins contained significant amounts of anti-insulin antibodies which were detected by radioimmunoassay. The anti-insulin antibodies were detected in all of the semi-synthetic insulins except for the WAT, 82-Hoc-L-Arg-His (N, N-dipropyl)-L-Arg-His (L, L-Arg-His) derivative.

Table I. Characteristics of Semi-Synthetic Insulins

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Anti-insulin Antibodies</th>
<th>Activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAT</td>
<td>0.43</td>
<td>38.5</td>
</tr>
<tr>
<td>82-Hoc-L-Arg-His (N, N-dipropyl) (L, L-Arg-His)</td>
<td>0.43</td>
<td>38.5</td>
</tr>
<tr>
<td>82-Hoc-L-Arg-His (N, N-dipropyl) (L, L-Arg-His)</td>
<td>0.43</td>
<td>38.5</td>
</tr>
</tbody>
</table>

The semi-synthetic insulins were found to be similar in their biological activity. However, the WAT, 82-Hoc-L-Arg-His (N, N-dipropyl)-L-Arg-His (L, L-Arg-His) derivative was found to be significantly less active than the other two derivatives.

CONCLUSION - The semi-synthetic insulins were found to be similar in their biological activity. However, the WAT, 82-Hoc-L-Arg-His (N, N-dipropyl)-L-Arg-His (L, L-Arg-His) derivative was found to be significantly less active than the other two derivatives.

REFERENCES

The results as presented in Table 11 showed that the recoveries of glycine and lysine from the three insulin derivatives treated with FDNB after acid hydrolysis were decreased by about 10% due to each component of the derivatized amino acids, whereas the recovery of glycine was the same in the FDNB-treated and untreated samples. Thus the amino group of all glycine and the amino group of 60% of lysine in the three derivatives were all free to react with FDNB and were therefore unaffected. The amino group of 81% phenylalanine was unreactive for reaction with FDNB and was therefore the site of selective chemical modification. In the case of the histidine derivative, the unreactive histidine residue is the only form of the amino acid residue that was unaffected by the derivatization procedure. This could be due to the fact that the histidine residue of the histidine derivative is the only form of the amino acid residue that was not derivatized by FDNB.

Table 11. Amino Acid Analyses of Insulin Derivatives With (+) and Without (-) FDNB Treatment.

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Number of Residues with (+) FDNB Treatment</th>
<th>Number of Residues with (-) FDNB Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.07</td>
<td>1.07</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.06</td>
<td>1.06</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Proline</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Valine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Based on Arginine = Glutamic acid = 10; on average of two analyses, not corrected for time-dependent destruction.

Theoretical values, taken from Ryle et al. (43).

Amino acid residues of interest.

The results as presented in Table 11 showed that the recoveries of glycine and lysine from the three insulin derivatives treated with FDNB after acid hydrolysis were decreased by about 10% due to each component of the derivatized amino acids, whereas the recovery of glycine was the same in the FDNB-treated and untreated samples. Thus the amino group of all glycine and the amino group of 60% of lysine in the three derivatives were all free to react with FDNB and were therefore unaffected. The amino group of 81% phenylalanine was unreactive for reaction with FDNB and was therefore the site of selective chemical modification. In the case of the histidine derivative, the unreactive histidine residue is the only form of the amino acid residue that was unaffected by the derivatization procedure. This could be due to the fact that the histidine residue of the histidine derivative is the only form of the amino acid residue that was not derivatized by FDNB.

In conclusion, the results of this study indicate that the derivatization procedure with FDNB can be used to selectively modify the amino acid residues of insulin. The recoveries of glycine and lysine from the three insulin derivatives treated with FDNB after acid hydrolysis were decreased by about 10% due to each component of the derivatized amino acids, whereas the recovery of glycine was the same in the FDNB-treated and untreated samples. Thus the amino group of all glycine and the amino group of 60% of lysine in the three derivatives were all free to react with FDNB and were therefore unaffected. The amino group of 81% phenylalanine was unreactive for reaction with FDNB and was therefore the site of selective chemical modification. In the case of the histidine derivative, the unreactive histidine residue is the only form of the amino acid residue that was unaffected by the derivatization procedure. This could be due to the fact that the histidine residue of the histidine derivative is the only form of the amino acid residue that was not derivatized by FDNB.

**REFERENCES**


**Biosynthetic Properties of the Insulin Derivatives**

The biosynthetic properties of the insulin derivatives were examined by the in vitro assay, receptor binding to their plasma membrane and radioligand assay. Based on the data from these assays, the three derivatives have been shown to be effective in vivo for the treatment of insulin-resistant states in animals. The results of these assays were consistent with the findings obtained from the in vitro studies, indicating that the insulin derivatives are effective in vivo for the treatment of insulin-resistant states.
Structure and function relationships of insulin. Preparation, characterization, and biological activity of three bovine insulin derivatives selectively modified at the NH2-terminal of the B chain.

C W Yeung, M L Moule and C C Yip