INTRAMOLECULAR CROSS-LINKED INSULIN

D.G. LINDSAY

Department of Biochemistry, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, Sussex, UK

Received 11 September 1971

1. Introduction

The use of bifunctional reagents for the preparation of inter- or intramolecular cross-links in proteins has been extensively used, particularly for information about inter-residue distances in proteins, for evaluating the importance of secondary forces in the stabilisation of protein structure, and for studying protein sub-unit interactions [1].

In extending my previous work on the modification of the amino groups of insulin with acylating agents [2], it was of interest to study the reaction of insulin with bifunctional acylating agents in order to obtain more information about the molecular architecture of the insulin molecule. It is pertinent to examine such derivatives in relation to the tertiary structure of insulin as shown in the crystallographic analysis of rhombohedral 2Zn insulin crystals [3] and to examine the effect such modification might have on the hypoglycaemic action of the hormone.

Bifunctional acylating agents have not been widely used for controlled cross-linking in proteins. Schnell and Zahn [4] have utilized the nitrophenyl esters of dicarboxylic acids to cross-link collagen. However, recent work [2] suggests that the use of the highly activated hydroxysuccinimide esters developed by Anderson et al. [5] for peptide synthesis, allows the formation of N-acyl derivatives of insulin in high yields using a 1:1 molar ratio of reagent to protein. They also appear to be highly selective for amino groups. By these means we have prepared a pure intramolecular cross-linked derivative of insulin by reaction with bis-hydroxysuccinimide succinate.

2. Materials and methods

Bis-hydroxysuccinimide succinate (m.p. 260° dec.) was prepared by a modification of the method of Schnell and Zahn [4] using N-hydroxysuccinimide instead of p-nitrophenol. The derivative was analytically pure.

Bovine insulin, essentially free of proinsulin, was dissolved in anhydrous dimethylformamide at a concentration of 0.1% w/v. Redistilled anhydrous triethylamine was added to the insulin solution at a concentration of 0.05% v/v. The mixture was then treated with bishydroxysuccinimide succinate (1 mole equivalent) in aliquots in dimethylformamide solution. The reaction mixture was left overnight. The dimethylformamide was removed on a rotary evaporator at 40° under reduced pressure and the product extensively dialysed against 0.01 M NH_4OH and lyophilised.

The modified insulin preparation was subjected to DEAE-Sephadex chromatography in 7 M urea buffers as previously described [2].

Gly^{A1}-hemi-succinyl-insulin was prepared by treatment of insulin with succinyl chloride in dimethylformamide solution (pract.) followed by isolation of the relevant isomer by chromatography on DEAE-Sephadex.

Oxidative sulfitolysis was performed by the method of Bailey and Cole [6] and the S-sulphonates separated by thin layer electrophoresis on cellulose plates using 4 M urea, 2.4 M formic acid buffer, 50 V/cm, i = 30 mA, t = 40 min. The derivatives were visualised by the Pauly stain.

The blood sugar assays were determined in rabbits previously fasted for 24 hr prior to an i.v. injection of insulin and derivatives. Each value was the mean of six animals and the initial mean value was taken as 100%. Insulin was injected at a dose of 0.5 IU/kg (0.0185 mg/kg) and the derivatives at a dose of 0.0185 mg/kg. The activity of the insulin and derivatives was determined from the blood glucose level after 30 min.

3. Results

Chromatography of the crude products of the reaction of insulin with bis-hydroxysuccinimide succinate is shown in fig. 1. Fractions 100–142 were rechromatographed under exactly the same conditions until a pure disubstituted insulin derivative was isolated as judged by isoelectric focussing [7]. Lindsay and Shall [2] have previously shown that disubstituted A^1, B^{29}-insulin derivatives are eluted in these fractions.

End group analysis [8] of the derivative showed that the gly^{A1} and lys^{B29} amino functions of insulin were modified. Treatment of the derivative with TPCK-treated trypsin [9] showed the presence of 0.11 mole of alanine per mole of insulin, suggesting that the lys B^{29} group of insulin was modified to the extent of 89%.

Oxidative sulfitolysis of the derivative and electrophoresis of the products (fig. 2) proved that the derivative was intramolecularly cross-linked. The derivative was also chromatographed on a Sephadex G-50 column using 7 M urea buffers [10] and eluted in the same position as insulin under these conditions and accordingly the material was designated as gly^{A1}-lys^{B29}-succinyl-insulin (fig. 3).

Biological testing of this material by the rabbit blood-sugar assay showed that it had approximately 60% of the biological activity of insulin assayed under the same conditions. This result was not significantly different from the activity of gly^{A1}-hemi-succinyl-insulin which also showed 60% of the biological activity of native insulin.

4. Discussion

Zahn and Meienhofer [11] studied the cross-linking of insulin with 1,5-difluoro-2,4-dinitrobenzene and demonstrated intramolecular cross-linking of gly^{A1} and lys B^{29} occurred at a concentration of 0.03% w/v. They concluded that these two groups in insulin must be $\simeq$ 5 Å apart for such a derivative to be favoured. However, a pure product was not isolated from these studies. We are able to report on the isolation of a pure intramolecular cross-linked insulin which retains a substantial amount of the biological activity of insulin.

Although uncertainties exist about the exact interpretation of the electron density map of insulin in the region of the terminal part of the B-chain [3], it seems certain that the gly^{A1} and lys^{B29} amino groups are $\simeq$ 5–8 Å apart in insulin crystals. Attempts are being made to crystallise this derivative to obtain further information about the effect the succinyl bridge has

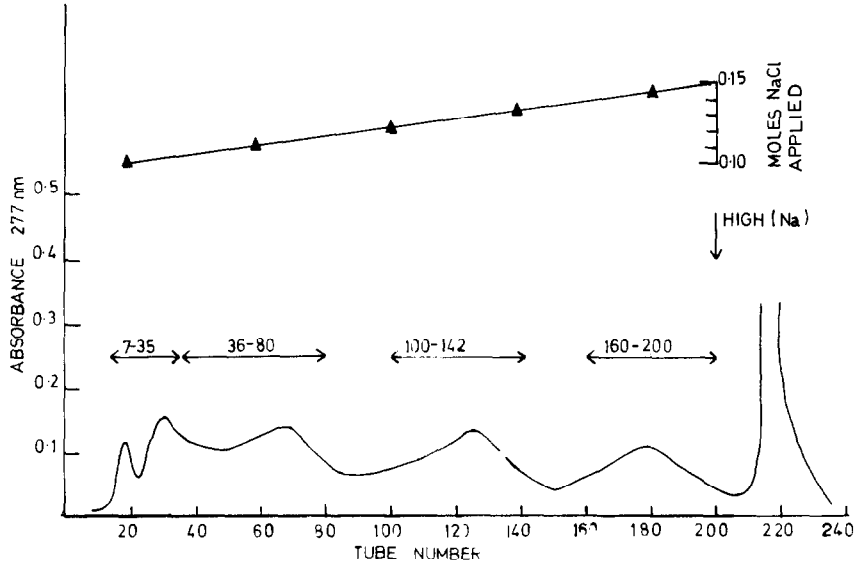

Fig. 1. DEAE-Sephadex chromatography of the reaction products of the modified insulin preparation (400 mg) using a column (2.5 cm × 40 cm) equilibrated with 7 M urea, 0.01 M Tris-HCl, 0.10 M NaCl at pH 7.30. The flow rate was 54 ml/hr and 9.0 ml fractions were collected. (——): absorbance at 277 nm. (▲——▲): moles of NaCl applied as a salt gradient.

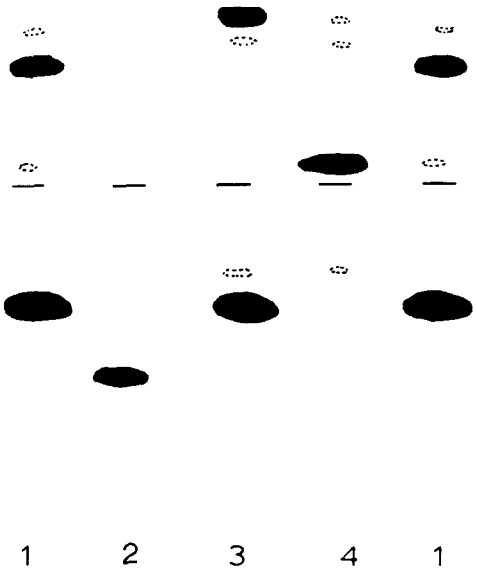

Fig. 2. Thin layer electrophoresis of sulfitolysed insulin and derivatives. (1) Sulfitolysed insulin. (2) gly^{A1}-lys^{B29}-succinyl-insulin. (3) Sulfitolysed gly^{A1}-hemi-succinyl-insulin. (4) Sulfitolysed gly^{A1}-lys^{B29}-succinyl insulin.

had on inter-atomic contacts in this area of the molecule. The linking of the terminal part of the B-chain by the succinyl bridge may help to evaluate the role of certain salt-bridges such as the A^{21}, B^{22} interaction in the stabilisation of the molecule.

It is of interest to compare the biological activity of gly^{A1}-lys^{B29}-succinyl-insulin with the activity of the acetoacetyl insulins previously prepared [2]. The activity of the cross-linked derivative is somewhat lower than the size of the succinyl group would indicate. This is not due to the diffent method of assay as acetyl insulins give exactly the same results when

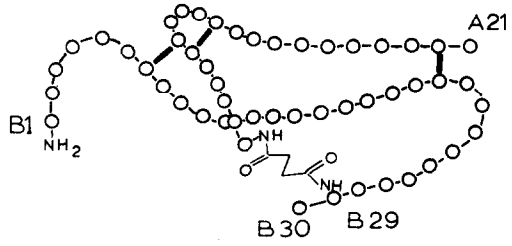

Fig. 3. Schematic representation of gly^{A1}-lys^{B29}-succinyl-insulin after the model of Zahn [16].

assayed by the mouse convulsion or the rabbit blood-sugar assay. It would imply that the rigidity of the succinyl bridge distorts the 'active site' of insulin. The activity of gly^{A1}-hemi-succinyl-insulin might be explained by repulsions caused by the introduction of a charged carboxyl group.

Gly^{A1}-lys^{B29}-succinyl-insulin can also be considered as a crude 'proinsulin-like' model. Although the connecting link joints the A^1 and B^{29} amino groups and not the amino group of A^1 and the carboxyl residue of B^{30} as in proinsulin, B^{29} and B^{30} occupy very close positions in the rhombohedral insulin crystal.

The profound influence of the connecting peptide of proinsulin on the ribosomal synthesis of native insulin has been demonstrated *in vitro* by Steiner and Clark [12] who reduced and subsequently reoxidised proinsulin and proved that the correct pairing of cysteine residues had occurred. However, it is possible to imagine that a smaller connecting bridge between glycine A1 and the terminal part of the B-chain of the insulin molecule is sufficient to ensure such correct pairing, particularly as the sequences of the connecting peptides of porcine [13], bovine [14], and human proinsulin [15] have shown that the connecting peptides which join the A1 and B30 residues in the insulin molecule are highly species variable, varying in both their length and in many amino acid substitutions. Recombination studies are in progress on a series of intramolecularly cross-linked insulins of varying chain lengths in order to test this hypothesis.

Acknowledgements

I would like to record my appreciation to the Royal Society for the award of a Stothert Fellowship and Schering AG, Berlin for their generous support of this work. I would also like to thank Drs. Losert and Loge, of Schering AG for the rabbit blood-sugar assays. Finally, I thank my colleague, Dr. S. Shall for his interest and encouragement.

References

[1] F. Wold, in: Methods in Enzymology, Vol. XI, ed. C.H.W. Hirs (Academic Press, New York, 1967) p. 617.
[2] D.G. Lindsay and S. Shall, Biochem. J. 121 (1971) 737.

[3] T.L. Blundell, J.F. Cutfield, S.M. Cutfield, E.J. Dodson, G.G. Dodson, D.C. Hodgkin, D.A. Mercola and M. Vijayan, Nature 231 (1971) 506.
[4] J. Schnell and H. Zahn, Kolloid-Zeitschrift und Zeitschrift für Polymers 203 (1965) 27.
[5] G.W. Anderson, J.E. Zimmerman and F.M. Callahan, J. Amer. Chem. Soc. 86 (1964) 1839.
[6] J.L. Bailey and R.D. Cole, J. Biol. Chem. 234 (1959) 1733.
[7] C. Wrigley, Science Tools 15 (1968) 17.
[8] W.R. Gray, in: Methods in Enzymology, Vol. XI, ed. C.H.W. Hirs (Academic Press, New York, 1967) p. 139.
[9] D. Levy and F.H. Carpenter, Biochemistry 6 (1967) 3559.
[10] R.W. Boesel and F.H. Carpenter, Biochem. Biophys. Res. Commun. 38 (1970) 678.
[11] H. Zahn and J. Meienhofer, Makromoleculare Chem. 26 (1958) 153.
[12] D.F. Steiner and J.L. Clark, Proc. Natl. Acad. Sci. U.S. 60 (1968) 622.
[13] R.E. Chance, R.M. Ellis and W.W. Bromer, Science 161 (1968) 165.
[14] D.F. Steiner, S. Cho, P.E. Oyer, S. Terris, J.D. Peterson and A.H. Rubenstein, J. Biol. Chem. 246 (1971) 1365. A. Salokangas, D.G. Smyth, J. Markussen and F. Sundby, European J. Biochem. 20 (1971) 183.
[15] P.E. Oyer, S. Cho, J.D. Peterson and D.F. Steiner, J. Biol. Chem. 246 (1971) 1375. A.S.C. Ko, D.G. Smyth, J. Markussen and F. Sundby, European J. Biochem. 20 (1971) 190.
[16] H. Zahn, VIth Intern. Congress Biochem., New York, N.Y., August, 1964.